REMARKS/ARGUMENTS

Status of the Claims

Upon entry of the present amendment, claims 1, 3-5 and 7-23 are pending. Claims 1, 3-4 and 11-19 are withdrawn as directed to a non-elected invention. Claims 5, 9-10 and 20 are amended. New claim 23 is added.

Claim 5 is amended to set forth an isolated peptide consisting of a peptide having an amino acid sequence of the recited SEQ ID NOs. Support is found, for example, in Table 4 on page 18.

Claims 9 and 10 are amended to set forth a pharmaceutical composition. Support is found, for example,

Claim 20 is amended to depend only from claim 5.

New claim 23 finds support, for example, in Examples 18 and 19 on page 25, line 31 through page 26, line 4 and in Table 8 on page 27, lines 21-28.

No new matter is added by the present amendments, and the Examiner is respectfully requested to enter them.

Restriction Requirement

With respect to the restriction requirement, Applicants reconfirm their election of the invention of Group 2 and submit that claims 5, 7-10, and 20-23 read on this invention.

With respect to the election of species, Applicants elect SEQ ID NO:30.

Claim Objections

Recitation of non-elected species

With respect to the Examiner's objection to claims 5, 8, 9, 10, and 20 for reciting non-elected embodiments (and implied instruction to cancel such non-elected embodiments from claim 5), Applicants submit that they are entitled to further consideration of non-elected embodiments upon an indication of allowance of the elected embodiment. Accordingly, the Examiner's objection is misplaced.

In particular, Applicants direct the Examiner's attention to M.P.E.P. § 803.02, which states that:

> **"If the members of the Markush group are sufficiently few in number or so closely related that a search and examination of the entire claim can be made without serious burden, the examiner must examine all claims on the merits, even though they are directed to independent and distinct inventions." (emphasis added)**

In a similar fashion, the Administrative Instructions to the PCT, Annex B, in addressing "Markush practice" in part (f), state that when a single claim defines chemical or non-chemical alternatives, the unity of invention requirement for a technical interrelationship and the same or corresponding special technical features shall be considered to be met when the alternatives are "of a similar nature". More particularly, when alternatives share (a) a common property or activity, and (b) a significant structural element or a common structure, they are deemed to be of a similar nature sufficient to demonstrate unity of invention.

In this case, the nonapeptides and decapeptides of SEQ ID NOs: 29, 30, 33, 34, 40, and 46 are identified herein as peptide fragments of VEGFR2 (KDR/flk-1; referred to below as KDR) having the common functional ability to bind to HLA antigens and induce cytotoxic T cells against KDR expressing cells, thereby making them useful as cancer vaccines for inhibiting angiogenesis. Accordingly, it is readily apparent that the recited peptides share a common utility. In addition, in that each of the recited polypeptides is a fragment of the KDR sequence, they also share significant common structure that is essential to this shared activity. Thus, in that the alternative sequences recited in claim 5 share a common activity as well as significant common structure, Applicants respectfully submit that they are so "closely related" or of such a "similar nature" that search and examination of the entire claim is warranted.

In this case, given the related nature of SEQ ID NOs: 29, 30, 33, 34, 40, and 46 discussed in detail above, Applicants submit that no undue burden would be imposed on the Examiner to conduct a computer search for each of SEQ ID NOs: 29, 33, 34, 40, and 46 together with elected SEQ ID NO: 30. Accordingly, Applicants respectfully request reconsideration and withdrawal of the election of species.

In any event, Applicants respectfully request the examination of the additional (non-elected) species upon an indication of allowability of the elected species pursuant to

M.P.E.P. § 803.02. In particular, it is noted that "should no prior art be found that anticipates or renders obvious the elected species, the search of the Markush-type claim will be extended [to the non-elected species]. . . The prior art search will be extended to the extent necessary to determine patentability of the Markush-type claim." M.P.E.P. § 803.02.

Pharmaceutical

The Examiner also objected to claims 9 and 10 for reciting "pharmaceutical" without the term "composition." The Examiner alleges that the term "pharmaceutical is an adjective and not a noun. Applicants do not agree with the Examiner. Applicants submit that the term "pharmaceutical" can be either an adjective or a noun. *See, e.g.*, definitions for "pharmaceutical" provided by dictionary.com.[1] However, in the interest of furthering prosecution, Applicants have amended claims 9 and 10 to recite a "pharmaceutical composition."

Objections to the Disclosure

The Examiner objected to the specification for containing embedded hyperlinks. In response, Applicants have removed the embedded hyperlinks in the specification.

Rejection under 35 U.S.C. § 101

Claims 5, 7-10, and 20-22 stand rejected under 35 U.S.C. § 101 as being directed to non-statutory subject matter. According to the Examiner, amending the claims to recite an "isolated" nonapeptide would obviate the rejection. Applicants do not agree with the Examiner. However, in the interest of expediting prosecution, is amended to set forth an isolated nonapeptide or decapeptide. Accordingly, withdrawal of this rejection is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph, enablement requirement

Claims 5, 7-10, and 20-22 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. The Examiner concedes that the specification enables an isolated nonapeptide ***consisting of*** the amino acid sequence of

SEQ ID NO: 30 that binds to HLA-A 0201 and induces high CTL activity. *See*, page 7 of the present Office Action.

Solely in the interest of expediting prosecution, Applicants have replaced the open language "comprising" with the closed language "consisting of" and limited the number of amino acid residues to be substituted or added to "one or two." While this amendment should not be construed as Applicants' agreement with or acquiescence to the Examiner's position regarding the alleged lack of enablement, Applicants nevertheless submit that such an amendment renders moot the Examiner's concerns regarding enablement of the claimed invention.

Moreover, Applicants submit that one of ordinary skill in the art would readily recognize how to make and use the invention presently claimed (*e.g.*, nonapeptides and decapeptides consisting of SEQ ID NOs: 29, 30, 33, 34, 40, and 46, optionally have one or two substitutions or additions) from the disclosures in the present specification coupled with information known in the art without undue experimentation and with a reasonable expectation of success. For example, on the issue of peptide analogues having one or two amino acid substitutions, it was well known in the art at the time of filing of the present application that the HLA-A2 binding affinity of epitope peptides could be maintained or enhanced after replacement at position 2 or C-terminal anchor residues (*see*, *e.g.* page 7, lines 14-17 of the present specification and Rammensee *et al.*, *Immunogenetics*, 1995, 41: 178-228 (for example, page 193, Table 2B); Kubo *et al.*, *J. Immunol.*, 1994, 152:3913-3924 (for example, right column of page 3915, lines 6-12 from the bottom); and Falk *et al. Nature* 1991, 351: 290-296 (for example, left column of page 293, lines 6-7 and Table 4 (not cited)).

In addition, Zaremba *et al.* in Cancer Res. 1997, 57:4570-4577 (for example, left column of page 4572, lines 4-10 and line 21 to right column, line 1 (not cited)) demonstrate that the CTL inducibility of an epitope peptide can be maintained or enhanced by the replacement of an amino acid residue at any one of positions 5-7 in the original epitope peptide. Further, it is reported that epitope peptides also maintained their CTL inducibility after substitution of amino acid residue at various positions in several publications. *See*, *e.g.*, Hoffmann *et al.*, *J Immunol.*

[1] Defintions for "pharmaceutical" provided by dictionary.com are attached as Appendix A.

2002, 168(3):1338-47 (for example, page 1344 2nd paragraph of Discussion); Dionne *et al.*, *Cancer Immunol Immunother.* 2003, 52: 199-206 (for example, right column of page 204, 4th paragraph); and Dionne *et al.* Cancer Immunology, Immunotherapy 2004, 53, 307-314 (for example, right column, 1st and 2nd paragraph).[2]

In the present application, the maintained CTL inducibility of a peptide analogue of which one amino acid residue is substituted at the N-terminal (SEQ ID NO: 54) was confirmed (*see*, *e.g.*, page 26, [Example 19] and Fig. 14).

```
V I A M F F W L L  ;SEQ ID NO: 30
  |
V L A M F F W L L  ;SEQ ID NO: 54
```

As for peptide analogues having one or two additional amino acid residues, it is generally accepted that such peptides will be digested in antigen presenting cells into nona- or deca- peptides, and will then bind with HLA to be presented on the surface of the cells. Thus, the resulting peptides which are presented in a HLA complex are the same as the original peptides. Accordingly, at the time of filing, once epitope peptides whose CTL inducibility has been confirmed are provided, a skilled artisan can obtain peptide analogues with maintained CTL inducibility by replacing or adding one or two amino acid residues to those epitope peptides without undue experimentation and with a reasonable expectation of success.

The Examiner is respectfully reminded that the test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, provided it is merely routine. *See*, *In re Wands* 8 U.S.P.Q. 1400, 1404 (Fed. Cir. 1988). In this case, Applicants submit the "trial and error" testing needed to identify regions of the peptide that can tolerate mutation is within the parameters of routine experimentation and optimization.

With respect to the Examiner's concerns regarding the unpredictability of "prevention" in the context of claim 9 on page 9 of the present Office Action, Applicants submit the Examiner's interpretation of the term "prevent" is unduly narrow. According to

[2] The cited references of Rammensee *et al.*, *Immunogenetics*, 1995, 41: 178-228; Kubo *et al.*, *J. Immunol.*, 1994, 152:3913-3924; Falk *et al. Nature* 1991, 351: 290-296; Zaremba *et al.*, *Cancer Res.* 1997, 57:4570-4577; Hoffmann *et al.*, *J Immunol.* 2002, 168(3):1338-47; Dionne *et al.*, *Cancer Immunol Immunother.* 2003, 52: 199-206; and Dionne *et al. Cancer Immunology, Immunotherapy* 2004, 53, 307-314 are attached as Appendix B.

www.wikipedia.org, "in medicine, prevention is any activity which reduces the burden of mortality or morbidity from disease". Prevention can occur "at primary, secondary and tertiary prevention levels." While primary prevention avoids the development of a disease, secondary and tertiary levels of prevention encompass activities aimed at preventing the progression of a disease and the emergence of symptoms as well as reducing the negative impact of an already established disease by restoring function and reducing disease-related complications.[3]

Accordingly, Applicants respectfully submit that, contrary to the Examiner's implication, the term "prevent," when afforded its ordinary and customary meaning, does not necessarily equate to absolute cessation. Moreover, Applicants respectfully submit that one skilled in the art would readily recognize that, in the context of the instant claims, prevention encompasses a wide range of prophylactic therapies aimed at alleviating the severity of the particular disorder, *e.g.* reducing the proliferation and metastasis of tumors, reducing angiogenesis, etc. Accordingly, Applicants submit that one of ordinary skill in the art would be able to "treat and prevent" tumors in accordance with the method of claim 9 without undue experimentation.

In view of the foregoing, the Examiner is respectfully requested to withdraw the present rejection.

Rejection under 35 U.S.C. § 112, first paragraph, written description requirement

Claims 5, 7-10, and 20-22 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the written description requirement. The Examiner concedes that the specification describes an isolated nonapeptide ***consisting of*** the amino acid sequence of SEQ ID NO: 30 that binds to HLA-A 0201 and induces high CTL activity. *See*, pages 10-11 of the present Office Action.

Solely in the interest of expediting prosecution, Applicants have replaced the open-end language "comprising" with the close-end language "consisting" and limited the number of amino acid residues to be substituted or added to "one or two". While this amendment should not be construed as Applicants' agreement with or acquiescence to the

[3] The entry for "prevention" from wikipedia.org is attached as Appendix C.

Examiner's position regarding the alleged lack of written description, Applicants nevertheless submit that such an amendment renders moot the Examiner's concerns regarding written description of the claimed invention.

Moreover, for the reasons discussed above, Applicants submit that one of ordinary skill in the art would readily recognize that Applicants were in possession of the invention presently claimed (e.g., nonapeptides and decapeptides consisting of SEQ ID NOs: 29, 30, 33, 34, 40, and 46, optionally have one or two substitutions or additions) from the disclosures in the patent coupled with information known in the art.

In view of the foregoing, Applicants respectfully maintain that those skilled in the art would recognize that Applicants have conveyed possession of the invention as claimed. Accordingly, the Examiner is respectfully requested to withdraw the present rejection.

Rejections under 35 U.S.C. § 102(b)

Flamme

The Examiner has rejected claims 5 and 8 under 35 U.S.C. § 102(b) as allegedly anticipated by Flamme, *et al.*, *Developmental Biol* (1995) 169:699-712 ("Flamme"). Applicants do not agree with the Examiner's position. However, in the interest of furthering prosecution, Applicants have amended claim 5 to set forth an isolated nonapeptide or decapeptide ***consisting of*** a peptide having the amino acid sequence of SEQ ID NO:30 or consisting of a peptide with cytotoxic T cell inducibility, wherein one or two amino acids have been substituted or added to the amino acid sequence of SEQ ID NO:30. Flamme does not disclose or suggest an isolated nonapeptide or decapeptide ***consisting of*** a peptide having the amino acid sequence of SEQ ID NO:30 or consisting of a peptide with cytotoxic T cell inducibility, wherein one or two amino acids have been substituted or added to the amino acid sequence of SEQ ID NO:30. Because Flamme does not disclose or suggest each and every element of the claimed peptide, Flamme does not anticipate the present invention. Accordingly, the Examiner is respectfully requested to withdraw the present rejection.

Kendall

The Examiner has rejected claims 5 and 8 under 35 U.S.C. § 102(b) as allegedly anticipated by U.S. Patent No. 5,712,380 ("Kendall"). Applicants do not agree with the Examiner's position. However, in the interest of furthering prosecution, Applicants have amended claim 5 to set forth an isolated nonapeptide or decapeptide ***consisting of*** a peptide having the amino acid sequence of SEQ ID NO:30 or consisting of a peptide with cytotoxic T cell inducibility, wherein one or two amino acids have been substituted or added to the amino acid sequence of SEQ ID NO:30. Kendall does not disclose or suggest an isolated nonapeptide or decapeptide ***consisting of*** a peptide having the amino acid sequence of SEQ ID NO:30 or consisting of a peptide with cytotoxic T cell inducibility, wherein one or two amino acids have been substituted or added to the amino acid sequence of SEQ ID NO:30. Because Kendall does not disclose or suggest each and every element of the claimed peptide, Kendall does not anticipate the present invention. Accordingly, the Examiner is respectfully requested to withdraw the present rejection.

Kubo

The Examiner has rejected claims 5 and 7-10 under 35 U.S.C. § 102(b) as allegedly anticipated by Kubo, *et al.*, *J Immunol* (1994) 152:3913-3921 ("Kubo"). Applicants do not agree with the Examiner's position. However, in the interest of furthering prosecution, Applicants have amended claim 5 to set forth an isolated nonapeptide or decapeptide ***consisting of*** a peptide having the amino acid sequence of SEQ ID NO:30 or consisting of a peptide with cytotoxic T cell inducibility, wherein ***one or two*** amino acids have been substituted or added to the amino acid sequence of SEQ ID NO:30. Kubo does not disclose or suggest an isolated nonapeptide or decapeptide ***consisting of*** a peptide having the amino acid sequence of SEQ ID NO:30 or consisting of a peptide with cytotoxic T cell inducibility, wherein ***one or two*** amino acids have been substituted or added to the amino acid sequence of SEQ ID NO:30. Because Kubo does not disclose or suggest each and every element of the claimed peptide, Kubo does not anticipate the present invention. Accordingly, the Examiner is respectfully requested to withdraw the present rejection.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

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8 results for: *pharmaceutical*

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Dictionary.com Unabridged (v 1.1) – *Cite This Source* – *Share This*

phar·ma·ceu·ti·cal [fahr-m*uh*-**soo**-ti-k*uh*l] Pronunciation Key – Show IPA Pronunciation

-adjective

1. pertaining to pharmacy or pharmacists.

-noun

2. a pharmaceutical preparation or product.

Also, **phar·ma·ceu·tic.**

[Origin: 1640–50; PHARMACEUTIC(S) + -AL[1]]

—Related forms

phar·ma·ceu·ti·cal·ly, *adverb*

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Appendix A

phar·ma·ceu·ti·cal (färˈmə-sōōˈtĭ-kəl) Pronunciation Key
adj. Of or relating to pharmacy or pharmacists.

n. A pharmaceutical product or preparation.

[From Late Latin `pharmaceuticus`, from Greek `pharmakeutikos`, from `pharmakeutēs`, *preparer of drugs*, variant of `pharmakeus`, from `pharmakon`, *drug*.]

phar'ma·ceu'ti·cal·ly *adv.*
(Download Now or Buy the Book)
The American Heritage® Dictionary of the English Language, Fourth Edition

Online Etymology Dictionary – *Cite This Source* – *Share This*
pharmaceutical

1648 (*pharmaceutic* in the same sense is from 1541), from L. *pharmaceuticus* "of drugs," from Gk. *pharmakeutikos*, from *pharmakeus* "preparer of drugs, poisoner," from *pharmakon* "medicine, poison." *Pharmacology* is attested from 1721, formed in Mod.L. (1683) with Gk. *-logia* "dealing with the topic of."

Online Etymology Dictionary, © 2001 Douglas Harper

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pharmaceutical

adjective
1. of or relating to pharmacy or pharmacists; "the pharmaceutical industry"
2. of or relating to drugs used in medical treatment

noun
1. drug or medicine that is prepared or dispensed in pharmacies and used in medical treatment

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American Heritage Stedman's Medical Dictionary – *Cite This Source* – *Share This*
phar·ma·ceu·ti·cal (färˈmə-sōōˈtĭ-kəl) or **phar·ma·ceu·tic** (-tĭk)
adj.

Of or relating to pharmacy or pharmacists.
n.
A pharmaceutical product or preparation.
The American Heritage® Stedman's Medical Dictionary

Merriam-Webster's Medical Dictionary – *Cite This Source* – *Share This*
Main Entry: **2pharmaceutical**
Variant: *also* **pharmaceutic**
Function: *noun*
: a medicinal drug

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Main Entry: **1phar·ma·ceu·ti·cal**
Pronunciation: "fär-m&-'süt-i-k&l
Variant: *also* **phar·ma·ceu·tic** /-'süt-ik/
Function: *adjective*
: of, relating to, or engaged in pharmacy or the manufacture and sale of pharmaceuticals <a *pharmaceutical* company> —**phar·ma·ceu·ti·cal·ly** /-i-k(&-)lE/ *adverb*

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Immunogenetics (1995) 41: 178–228

ANNIVERSARY REVIEW

Hans-Georg Rammensee · Thomas Friede
Stefan Stevanović

MHC ligands and peptide motifs: first listing

Received: 13 December 1994

Introduction

The purpose of this article is to provide a compendium of major histocompatibility complex (MHC) peptide motifs and MHC ligands known to date, together with a discussion of the methods used to gain this information. A problem here is the exponential growth of information in this field over the recent years. The number of known MHC ligands was zero in 1989 and three in 1990. This article, written in 1994, lists a couple of hundred such ligands, plus a large number of likely ligands. By the time this work is published, we expect a lot more ligands to be known. On the other hand, the peptide motifs of many of the more important MHC class I molecules are known already, so that this article will still be useful as a source of information. For class II, the situation is a bit different. Only a few allele-specific motifs have been reported, and the data from different authors are partially conflicting. The principles of allele-specific ligand motifs, however, have emerged recently by the combination of information on MHC class II structure, ligand sequencing, and peptide binding assays. Thus, these principles can be applied to further ligands to be identified.

Overview of MHC function

MHC molecules are peptide receptors. Their function is to collect peptides inside the cell and to transport them to the cell surface, where the complex of peptide and MHC molecule may be recognized by the T-cell receptor (TCR) for antigen of T lymphocytes (Rammensee et al. 1993). In normal cells, MHC-associated peptides are derived from normal, that is, self proteins. During their differentiation, T cells become tolerant to complexes of self peptides and self MHC molecules (Von Boehmer 1992). Thus, if any new peptides, e.g., derived from an infectious agent, occur later, they can be recognized by T cells. Since the specific immune system is regulated by T cells, the trimolecular complex of TCR, MHC molecule, and peptide can be considered a control switch for the immune system. Thus, a study of the molecular interactions between the three parts is essential for our understanding of the immune system.

Two classes of MHC molecules are distinguished, class I and class II. Class I molecules consist of a membrane-inserted heavy chain of about 45000 M_r, and a non-covalently attached light chain of 12000 M_r (Klein 1986). The latter is also known as β_2-microglobulin (β_2m). The structure of class I molecules has been resolved by X-ray crystallography (Stern and Wiley 1994). It has some resemblance to a moose's head, whereby the antlers would form a groove that is recognized as a peptide-binding device. HLA-A, B, and C are the "classical" class I molecules of humans, and H-2K, H-2D, and H-2L those of the mouse. Class II molecules are heterodimers consisting of two chains α and β, of similar size (about 30000 M_r), both of which are membrane inserted. HLA-DR, DQ, and DP are the human class II molecules, H-2A and E those of the mouse. Their structure is surprisingly similar to that of class I molecules (Stern and Wiley 1994; Stern et al. 1994; Brown et al. 1993).

All HLA molecules, including the numerous "non-classical", are encoded on chromosome 6, with the exception of β_2m which is on chromosome 15. *H2* genes are on chromosome 17 of the mouse, and the mouse β_2m gene is on chromosome 2.

A peculiarity of MHC genes is their extensive polymorphism, characterized by the presence of dozens or hundreds of alleles in a species. *H2* alleles are designated $H2K^b$, $H2K^d$, $H2K^k$ and so on for class I, and $H2Aa^b$, $H2Aa^k$, $H2Ab^k$, $H2Eb^d$ and so on for class II, whereby different alleles may differ in as many as 40 amino acids (Klein 1986). The present nomenclature (Bodmer et al. 1994) of *HLA* genes and products (which has been changed several times) is outlined as follows: class I heavy chain

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Appendix B

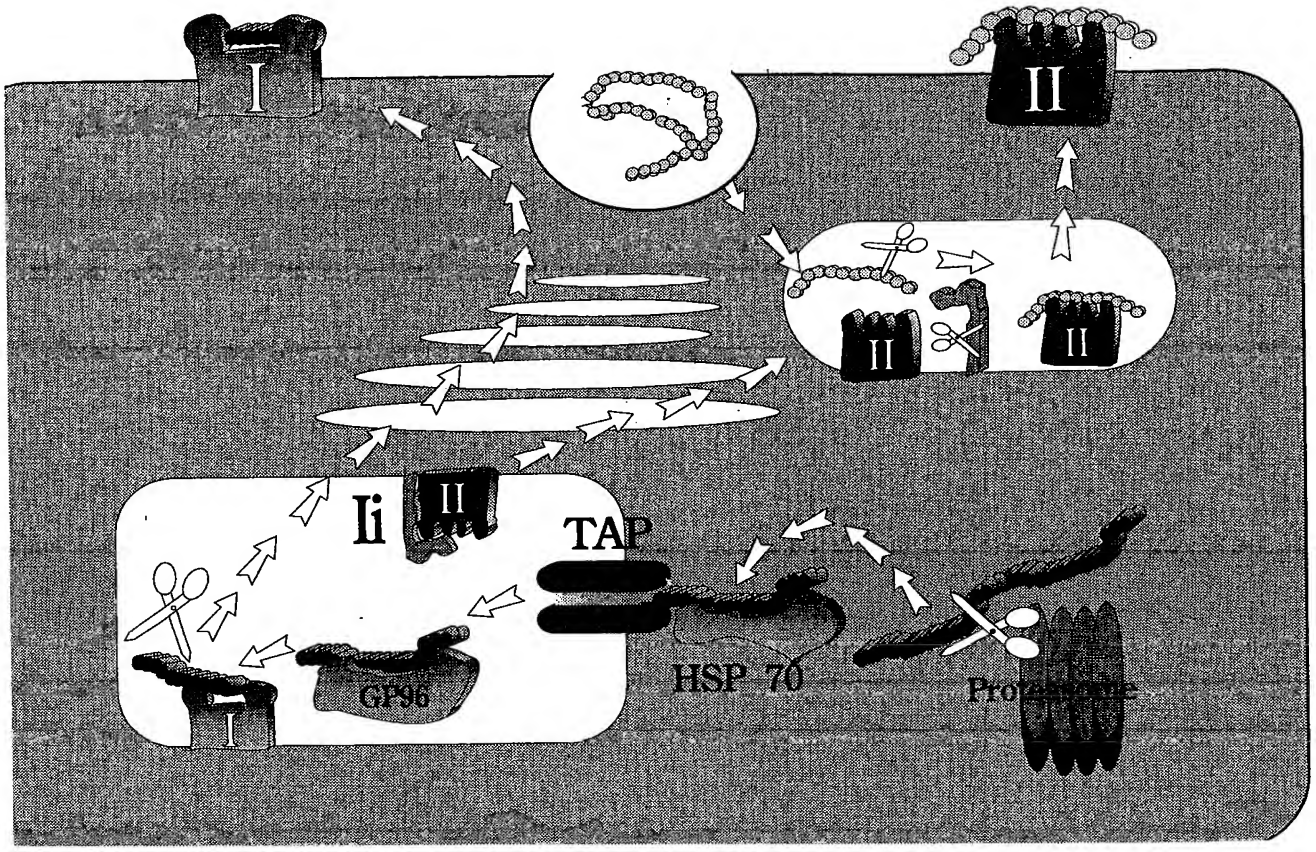

Fig. 1 A simplified and partially hypothetical overview of antigen processing. For explanation see text

loci: *HLA-A, B,* and *C*; class II α chain loci: e.g., *HLA-DRA, DQA1, DPA1*, class II β chain loci: e.g., *HLA-DRB1, DRB3, DQB1, DPB1*. Alleles are designated, for example, *HLA-A*0201*, or *HLA-DRB1*0101*. This nomenclature can only be applied if the respective sequences are known. Since this is not the case in many situations, the old designations, e.g., *HLA-A2* or *HLA-DR3*, based on serology, are still being used, and describe collections of alleles with shared serologic determinants (e.g., *HLA-A2* for *A*0201* through *A*02012*). Both class I light chains and HLA-DRα chains are not very polymorphic (Klein 1986). The high (*HLA-B*) or at least moderate polymorphism of the other genes results in the expression of a large number of combinations of alleles at the different loci per chromosome (haplotype), and in a high degree of heterozygosity. Thus each individual has his or her particular combination of HLA molecules, namely up to six different class I and about six different class II molecules (if the non-classical HLA molecules, whose function is not known, are not considered), making it unlikely to find two unrelated individuals with exactly the same combination of *HLA* genes.

A simplified outline of MHC function is given in the diagram in Figure 1. Class I molecules, both heavy and light chains, are synthesized into the ER (reviewed in Jackson and Peterson 1993). The peptides to be loaded on class I molecules are, in many cases, derived from cytosolic proteins. The details of peptide generation are not known definitely. A widely held view, however, is that cytosolic proteins are partially degraded by an endopeptidase activity of the proteasome, a multiunit structure with several activities located in the cytosol (Rock et al. 1994). It is not clear, however, how the products of such endopeptidase activity are related to the final class I ligands (Dick et al. 1994). One possibility is that the proteasomes directly produce the correct ligands. Alternatively, proteasomes could cut out larger peptides requiring further processing. The endopeptidase specificity of the proteasome is such that a protein is cut after hydrophobic or charged residues, in principle. The fine specificity of endopeptidase activity is influenced by two proteasome subunits, LMP2 and LMP7, which are encoded in the MHC region and regulated by IFN. However, the exact kind of LMP influence on specificity is controversial (Howard and Seelig 1993). In any case, such peptides must be transported into the ER lumen by the TAP molecule [(transporter associated with processing) (Neefjes and Momburg 1993)]. According to one hypothesis, these peptides are bound and protected from complete degradation by a chaperone, HSP70, before reaching TAP (Srivastava et al. 1994). Peptide transport by TAP molecules has

been directly demonstrated recently (reviewed in Momburg et al. 1994). Transport has specificity especially regarding the C-termini of peptides, and selectivity for peptide lengths. Peptides of 7 to 23 amino acids have been shown to be transported, whereby optima of 10 to 15 amino acids are seen. Human TAPs do not discriminate much between the C-termini of peptides. In contrast, the mouse TAP has a preference for peptides with hydrophobic C-termini and dislikes peptides with charged termini. This pattern of specificities fits well with the peptide specificities of human and mouse MHC class I molecules, since all mouse class I molecules require peptides with hydrophobic C-termini, whereas some human class I molecules require peptides with basic C-termini. On the other hand, mouse cells transfected with the *HLA-A3* gene, requiring peptide ligands with basic C-termini, can be loaded with the fitting peptides (Maier et al. 1994). This indicates that MHC peptide specificity need not be strictly dependent on TAP specificity. That TAP specificity indeed can influence MHC peptide loading is evident from two different TAP forms in the rat, TAP^a and TAP^u. Dependent on co-expressions of the respective TAP, the peptide spectrum of rat MHC class I molecules, $RT1^u$, is different, as indicated by different HPLC behavior of $RT1^a$-associated peptides. When measured in a peptide transporter assay, TAP^a has the same specificity as human TAP, that is, it does not discriminate between hydrophobic and basic C-termini, whereas TAP^u is more like the mouse transporter, with a preference for peptides with hydrophobic C-termini.

Once they are inside the ER lumen, the further fate of transported peptides is not exactly known. The recently reported physical association of TAP molecules and class I molecules suggested that peptides are directly loaded onto class I molecules immediately after discharge from the transporter (Ortmann et al. 1994; Suh et al. 1994). However, this would require that either the incoming peptides are already of the right size for loading to class I molecules, or that they bind as longer peptides (Falk et al. 1990) and are trimmed while somehow bound to MHC. An alternative hypothesis is that peptides are first bound by a chaperone, gp96, which shuttles the peptides to class I molecules, perhaps with some trimming of peptides underway. The main reason for assuming that gp96 plays a role in antigen processing stems from an impressive series of experiments by Srivastava and co-workers (1994), showing that gp96 molecules are associated with a large array of peptides and are able to immunize mice against antigens presented by MHC class I molecules.

In any event, the peptide somehow reaches the class I molecule and binds into the groove, perhaps after a final trimming step while already in touch with MHC. Unusually long peptides found associated with MHC class I molecules might have escaped such a final trimming (Urban et al. 1994). The assembly sequence of class I heavy chain, $\beta_2 m$ and peptide is not quite clear. A recent report indicates that another chaperone, calnexin, is bound to assembled complexes of heavy chain and $\beta_2 m$, and thus retains empty class I molecules in the ER (Jackson et al. 1994). It is only upon peptide loading that the fully assembled heavy chain/$\beta_2 m$/peptide complex is released by calnexin for transportation to the cell surface.

Class II molecules also start their existence in the ER. The two chains, α and β, assemble and are bound by a chaperone-like molecule, the invariant chain [(Ii) (Cresswell 1994)]. This molecule has two functions; one is to direct the α,β-heterodimer to the class II loading compartment, which appears to be a specialized vesicle characterized by the presence of class II molecules. The second function of Ii is the prevention of premature peptide loading to class II molecules. The molecular interactions between Ii and the α,β-heterodimer preventing peptide binding are not completely sorted out; one possibility is an allosteric effect of Ii on the dimer such that the peptide binding groove is closed due to conformational change. The other possibility is that a particular stretch of the invariant chain binds into the groove and thereby competitively prevents the binding of peptides. This latter view is derived from the observation that Ii peptides, called CLIPs (class II-associated invariant chain peptides) are frequently found associated with immunoprecipitated class II molecules, and that CLIPs are especially abundant in cells with a defect in antigen processing. In any case, Ii is removed from the α,β-heterodimer in the class II loading compartment, or shortly before. The peptides loaded onto class II molecules can be derived not only from endocytosed protein but also from protein endogenous to the cells, especially membrane-bound proteins which have a chance to co-localize in the class II loading compartment. Finally, the peptide-loaded α,β-heterodimers are translocated to the cell surface.

The simplified view shown in Figure 1 suggests a strict separation of the processing pathways for class I and class II, respectively. There is strong evidence, however, for considerable cross-talk between the two pathways. Peptides derived from cytosolic proteins, for example, can be loaded onto class II molecules (Pinet et al. 1994). On the other hand, peptides derived from phagocytosed proteins can be loaded onto class I molecules, especially if the phagocytosed protein is aggregated (Pfeifer et al. 1993; Rock et al. 1993). Such side-lines of processing pathways deserve interest because they could be exploited for new strategies of immune intervention.

Methods of characterizing MHC/peptide interactions

The most seminal approach to gain information on the function of MHC molecules as peptide receptors is the X-ray analysis of MHC crystals (Stern and Wiley 1994). The two other principle methods are: 1) Biochemical isolation and study of naturally MHC-associated peptides, and 2) Binding studies with synthetic peptides. The latter two approaches are discussed below:

1) Analysis of natural MHC ligands

The diagram in Figure 2 gives an overview on the approaches used for isolation and analysis of MHC-associated peptides.

The major technical challenge is the small copy number of individual peptides. It is estimated that a cell presents well over 1000 different peptides on its 100000 or so copies of a given MHC allelic product. A few of these peptides are present in high copy number, that is, up to 10000 or more. By far the most ligands, however, occur in a much lower copy number, maybe even down to as low as one copy per cell.

The most sensitive means of detecting isolated peptides is the T-cell assay, which is able to detect peptides in the sub-picomolar range, at least as far as cytotoxic T cells are concerned (Rötzschke et al. 1990). Typically, a peptide-containing sample (e.g., a few µl of an HPLC fraction) is incubated in a total volume of 100 µl together with MHC-expressing, ^{51}Cr-labeled target cells. After some incubation time, e.g., 90 min, CTL are added, the supernatant is harvested 4 to 6 h later, and the relative radioactivity measured indicates the degree of target cell lysis. If the 100 µl volume used for target cell incubation has a concentration of 1 pM, the absolute amount of peptide is 100 attomol, a sensitivity not reached by any other method. The use of the CTL assay, of course, is limited to the detection of T-cell epitopes for which T cells are on hand: Viral antigens, minor H antigens, tumor-associated antigens, transfected model antigens, or antigens recognized by alloreactive T cells. On the other hand, peptide detection assays for class-II-restricted T cells appear to be less sensitive than for class I-restricted T cells.

The major shortcoming of the T-cell assay for peptide detection is that it does not give sequence information. However, the location of a T-cell epitope among HPLC-separated MHC ligands of an infected cell can allow identification of the peptide in combination with biochemical analysis such as Edman degradation or mass spectrometry. The first naturally processed viral T-cell epitopes indeed were identified by the combination of T-cell assay with mass spectrometry, comparison of the HPLC behavior of synthetic and natural peptides, or partially direct sequencing, using radiolabeled amino acids incorporated by virus-infected cells (Rötzschke et al. 1990; van Bleek and Nathenson 1990). A combination of these methods for identification of T-cell epitopes is only possible if the proteins of origin are known. Direct sequencing of HPLC fractions containing a T-cell epitope is rarely successful, namely, only in cases where the T-cell epitope happens to be a peptide highly abundant in that fraction. A marked improvement of sensitivity was brought about by an ingenious combination of HPLC, CTL assay, and mass spectrometry by Cox and co-workers (1994).

By far the most ligands known to date are not T-cell epitopes and these ligands were determined by direct sequencing, either by Edman degradation, or by mass spectrometry, or by a combination of the two methods. Detection limit of Edman degradation is about 1 pmol, that

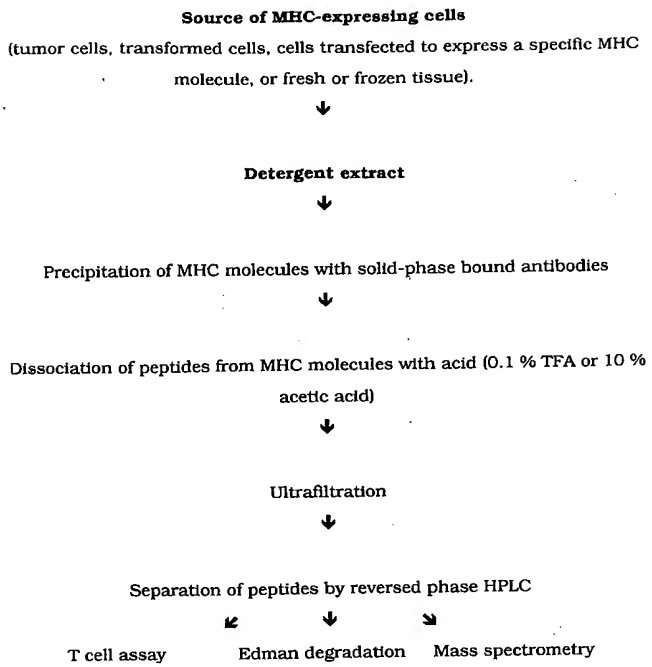

Fig. 2 Methods for analysis of MHC ligands

is, the equivalent of 6×10^9 cells for a peptide occurring in 100 copies per cell. Sequencing by tandem mass spectroscopy has been reported to be more sensitive – down to 30 fmol or less. It is, however, challenging to achieve this degree of sensitivity, so that, apart from the pioneering group of Hunt and co-workers (1992), not many other laboratories have come up with similar results.

A special application of Edman degradation is pool sequencing, that is, altogether-sequencing of the complex mixture of peptides eluted from a given MHC species (Falk et al. 1991b). Although disliked by purists, this approach allows one to determine the common characteristics of all peptides associated with a given MHC molecule, with relatively little effort. Pool sequencing of MHC class I ligands led to the discovery of the principle of allele-specific motifs, and allowed a large number of such motifs to be determined. The clear information that can be obtained from pool sequencing of class I ligands is made possible by their uniform length, e.g., 9 amino acids. But even for class II ligands, which can range in length from about 12 to 25 amino acids, pool sequencing is a valuable tool for gaining detailed information on motifs (Falk et al. 1994b).

It appears that the number of amino acids between the N-terminus of class II ligands and the first anchor varies by about three amino acids for the majority of ligands. For DR1, for example, the distance from the N-terminus to the first anchor of the majority of ligands is 5 ± 1 (Falk et al. 1994b). Thus, pool sequencing indicates a cluster at position 4, 5, and 6 for the anchor residues used, aromatic and aliphatic. Again for DR1, the next cluster stretches over

positions 7, 8, and 9, indicating the next anchor for aliphatic residues. The rough motif obtained by such interpretations – absolute position 5 set as relative position 1 to mark the first anchor – can then be complemented and worked out in depth by applying 1) alignment of natural ligands, 2) consideration of the pockets, as revealed recently by crystallography of a monopeptidic DR1 molecule (Stern et al. 1994), and 3) considerations of peptide binding assays. If all four sources of information are considered, a detailed picture of the degenerate (as compared with class I) peptide specificities of class II molecules can be obtained that should be useful for epitope predictions (Friede and co-workers, submitted).

2) Peptide binding assays

MHC/peptide binding assays have a history of leading to obsolete results. On the other hand, with our increasing knowledge of MHC structure and MHC/peptide interaction and specificity, it is possible to design straightforward peptide binding experiments to answer specific questions. A number of approaches can be used to measure peptide binding to MHC. The oldest method is as follows (Buus et al. 1987): MHC molecules are purified and incubated with radioactively labeled peptides. Then the mixture is subjected to a gel filtration column. MHC molecules with the radioactive peptide bound will elute in the exclusion volume, whereas free peptides will elute later. Thus, the amount of radioactivity in the exclusion volume is a measure for peptides bound to MHC. The binding behavior of other, unlabeled peptides can be tested via their capacity to inhibit binding of the radioactive peptide. A number of variations of this method have been used. For example, the radioactive label can be replaced by a fluorescent marker. Furthermore, MHC/peptide complexes can be separated from free peptides by gel electrophoresis, or upon binding of the MHC/peptide complex to solid phase with the help of antibodies. In the latter case, however, two different antibodies reactive with different sites of the MHC molecule are required, one for purification of the MHC molecule, the other for capturing the MHC/peptide complex from the reaction mixture.

Depending on the conditions, these kinds of peptide binding assays can be made very sensitive to detect even low-affinity peptide binding. This may result in problems of interpretations, since low-affinity binding might not be relevant for physiological MHC/peptide interactions.

A second type of binding assay depends on the stabilization of MHC class I molecules by bound peptides. Cells with a defect in antigen processing, for example, TAP-defective RMA-S cells, express only a low density of antibody-detectable MHC class I molecules on their surface, if cultured under normal conditions (37 °C). If such cells are incubated with peptides binding to the expressed class I molecules with high affinity, the latter are stabilized, and their surface density increases (Townsend et al. 1989). Since determination of class I surface density can be easily done by FACS analysis, this approach has been widely used. Since only few cell lines with transporter defects are known, the assay can only be used for MHC molecules expressed by such cells, e. g., H-2K^b or D^b for RMA-S cells. To study peptide binding for additional MHC-molecules, the desired MHC molecule can be expressed in RMA-S or other TAP-defective cells upon gene transfection. The advantage of this MHC-stabilization assay is that it is rather insensitive and thus detects only peptides binding with high affinity that are likely to be physiologically relevant. Stabilization of MHC molecules by peptides can also be measured with purified MHC molecules.

For class II molecules, the binding of high-affinity peptides leads to a compact form of the MHC/peptide complex, as seen by SDS gel electrophoresis, whereas a peptide of lower affinity leads to a "floppy" form of class II molecules.

A very elegant approach for studying the peptide specificity of class II molecules has been developed by Hammer and co-workers (Sinigaglia and Hammer 1994). A peptide library is expressed by bacteriophages. From the peptide-expressing phages only those are selected which are able to bind to a given class II molecule. The peptide sequences expressed by the selected phages are then determined. With this approach, a peptide binding motif of HLA-DR1 has been established that is well reflected among the natural ligands, and can be well explained by the crystal structure of HLA-DR1.

MHC class I ligands and motifs

The main purposes for which this information will be useful are the prediction of T-cell epitopes within proteins of known sequences and the detailed analysis of peptide/MHC interaction. For epitope prediction it is important not to consider just the basic motif of a given MHC molecule, since the non-anchor positions of peptides could also contribute considerably to the interaction with MHC. This is evident from the preferences seen for certain residues at non-anchor-positions in pool sequencing data, from the interaction of such residues with MHC sites as seen in crystals (Madden et al. 1993; Zhang et al. 1992; Fremont et al. 1992), and from detailed binding studies showing that certain residues at a given peptide position can be detrimental for binding (Ruppert et al. 1993; Kast et al. 1994; Parker et al. 1994).

The basic approach to search a protein sequence for an epitope fitting to a given class I molecule is as follows. First, the sequence is screened for stretches fitting to the basic anchor motif (2 anchors in most cases), whereby allowance should be made for some variation in peptide length as well as in anchor occupancy. If a motif, for example, calls for 9mers with I or L at the end, 10mers with a fitting C-terminus should be considered as well, and other aliphatic residues at the C-terminus, like Val or Met, should also be considered. In this way, a list of candidates will be obtained. These are now inspected for having as many non-anchor residues as possible in common with

ligands already known, or with the residues listed among the "preferred residues" or "others" on top of each motif Table. If possible, a binding assay can be performed at this stage to exclude weak binders which occur frequently among peptides conforming to a basic motif. If a detailed study on peptide binding requirements is available, the candidates can also be screened for non-anchor residues detrimental or optimal for binding (Ruppert et al. 1993; Kast et al. 1994; Romero et al. 1991; Ebert et al. 1993). One should keep in mind, however, that pure peptide binding motifs can be misleading in the search for natural ligands, since other constraints, such as enzyme specificity during antigen processing and specificity of transporters or chaperones, are likely to contribute to ligand identity in addition to the MHC binding specificity.

A careful consideration of the pocket structure of the MHC molecule of interest can also be useful for epitope prediction (Falk and Rötzschke 1993). For the P1 residue, for example, preferences can be explained by the residues contributing to the P1 contact site (Falk et al. 1995a,c). Since the MHC residues contributing to the different contact sites can differ among MHC molecules, such considerations should be held with caution, however (Guo et al. 1993). Computer modeling of the MHC molecule in question can be of help here.

The use of allele-specific peptide motifs is limited to a certain extent by exceptional ligands not fitting to a motif, e.g., Frumento and co-workers (1993) and Mandelboim and co-workers (1994). Such ligands will be missed by epitope predictions based on allele-specific motifs. It is not clear as yet how frequently this happens. In most cases, natural ligands will fit to the motifs, whereby substitutions of anchor residues with residues of similar chemistry (e.g., one aliphatic residue for another) and length variations are not infrequent and should be considered. A special case is the mouse H-2M3 molecule. A complete motif is not known, except that this molecule is specialized to present N-formylated peptides of bacterial or mitochondrial origin (Fischer-Lindahl 1991; Shawar et al. 1991).

MHC class II ligands and motifs

The long-awaited X-ray analysis of class II molecules has given us invaluable insight into peptide/class II interactions (Brown et al. 1993; Stern et al. 1994). Especially the detailed information on the 5 DR1-pockets accommodating anchoring side chains of one particular ligand, influenza haemagglutinin 306-318, provided a structural basis for the previously worked out peptide ligand motif of DR1 molecules (Rötzschke and Falk 1994; Sinigaglia and Hammer 1994). Moreover, pocket spacing and structure, as found for this one particular DR1/peptide complex, can be used to deduce the putative interaction for other DR1-peptide complexes and even for some other class II molecules. We found it particularly useful to evaluate pool sequencing data under the aspect of the expected pocket structure (Friede and co-workers, submitted; Schild and co-workers, submitted). Combined with the alignment of individual class II ligands, this approach is a powerful tool to determine allele-specific class II peptide motifs, as we have exercised recently for several closely related DR4 subtypes (Friede and co-workers, submitted).

The general picture for allele-specific class II motifs emerging is as follows. A stretch of nine amino acids, on average starting at absolute positions 3 to 5 of natural ligands, is determined by the respective allele-specific motif, corresponding to the peptide portion embedded in the MHC groove. The first position of this nonamer stretch, P1, represents a hydrophobic anchor for all class II ligand motifs known so far. Anchoring of the hydrophobic P1 side chain in the respective class II pocket appears to be particularly intensive, as impressively illustrated by the deep pocket seen in the monopeptidic DR1 crystal. The importance of P1 side chains is also indicated by the striking influence of P1 on peptide binding, and by the significant clustering of hydrophobic residues at cycles 3 to 5 of self-peptide pools. In addition to P1, several other anchors follow up to P9. For DR1, these are at P4, P6, P7, and P9, as indicated by structural data, whereby the specificity of P7 is somewhat degenerate and escapes detection in binding assays or natural ligand analysis. For several other class II molecules, the same anchor spacing – P1, P4, P6, P7, P9 – is compatible with ligand motif data. DR2, DR3, and DR4 motifs as well as H-2E motifs fall into this category. Other molecules, like DR5, DPw4, and DQ7 appear to have slightly different anchor spacing, e.g., the second anchor at P3, or an anchor at P5. Allele-specific differences can occur at each of the anchor positions, although differences of P1 specificity in HLA-DR molecules are limited by the β86Gly/Val polymorphism. More pronounced allele-specific differences are found for P4, P6, and P9, respectively. Charge differences are particularly evident; P4 of DR17, for example, requires Asp, whereas P4 of DR4Dw10 does not tolerate Asp or Glu but prefers basic or hydrophobic residues. P9, on the other hand, prefers hydrophobic residues for DR1 but negative charges for DR4Dw15 and positive charges for H-2E^k. Interestingly, charge differences in polymorphic stretches of class II molecules (probably reflecting counter charges for charged anchors) have been found to be associated with autoimmune diseases (Gregersen et al. 1987; Khalil et al. 1990; Todd et al. 1987).

Epitope prediction of class II ligands within a protein is not as easy as with class I, because the anchors, or interaction sites, are more degenerate in their specificity. The first step should be to pick out the most allele-specific anchor beyond P1, for example, P4 of DR17, P6 of DR1, or P9 of H-2E^k or DR4Dw15. The selection of nonamer sequences fitting to P1 and the other anchor of the respective motif is then further examined for adherence to the additional anchors. The resulting collection of nonamer stretches might then be inspected for adherence to the putative processing motif XPXX in the flanking regions (Rötzschke and Falk 1994). A quantitative ranking of the contribution of each amino acid residue at almost every position has been determined in an elegant approach by

Hammer and co-workers (1994) for DR4, which led to highly accurate predictions of good DR4 binders.

Technical notes

We have tried to put together all the motifs and natural ligands we were aware of. Due to the flood of data emerging in the past years, however, we anticipate that some information has been overlooked. We apologize in advance to those authors whose work was inadvertently not adequately covered.

In case of those class II ligands occurring as nested sets, we included only one or a few members of the set in many cases.

An X in peptide sequences stands for an undetermined amino acid. However, if the peptide sequence has been determined by mass spectometry, as is the case for the peptides reported by Hunt and co-workers (1992a, b), X stands for either Leu or Ile (which have the same mass). Lowercase letters in peptide sequences indicate residue determination of lower confidence.

As far as T-cell epitopes are concerned, only those have been selected which are likely to be naturally processed; criteria for judgement are to be found in Stevanović and Rammensee (1995). From the numerous class II motifs that have been published, we selected the more convincing ones, that is, those compatible with the class II structure. Due to the variable number of amino acids between the N-terminus and the first anchor of peptides, alignment of ligands or T-cell epitopes to class II motifs is always arbitrary, unless a structural analysis has been performed. For the class II molecules without reasonable motifs, a list of the published ligands is provided, without any attempt at alignment.

If you wish to have your motifs or ligands included in forthcoming listings, please send us reprints (no preprints) of the work describing them. We would also appreciate any comments on errors and omissions, as well as suggestions for improvements.

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Table 1 Mouse class I motifs
A H-2K^d

	Position												Source	Ref.
	1	2	3	4	5	6	7	8	9					
Anchor residues		**Y** **F**							**I** **L** **V**					a
Preferred residues			N I L	P	M	K F	T N							
Others	K A R S V T		A H V R S F E Q K M T	A E S D H N	V N D I L S T G	H I M Y V R L	P H D E Q S	H E K V F R						
Examples for ligands	T	**Y**	Q	R	T	R	A	L	**V***				Influenza A NP 147–154	b, c
	S	**Y**	F	P	E	I	T	H	**I**				Tyrosine kinase JAK1 355–363	a, d
	K	**Y**	Q	A	V	T	T	T	**L***				Tum-P198 14–22	a, e, f
	G	**Y**	K	D	G	N	E	Y	**I***				Lysteriolysin O 91–99	g
	K	**Y**	G	V	S	V	Q	D	**I***				L. monocytogenes p60 217–225	h
	G	**Y**	L	G	Q	V	T	X	**I**				Unknown	u
	S	**F**	V	D	T	R	T	L	**L**				Collagen 1 α2 4–12	u
T-cell epitopes	L	**Y**	Q	N	V	G	T	Y	**V**				Influenza JAP HA 204–212	i
	T	**Y**	V	S	V	G	T	S	**T**	**L**			Influenza JAP HA 210–219	l
	V	**Y**	Q	I	L	A	I	Y	**A**				Influenza JAP HA 523–531	i
	I	**Y**	A	T	V	A	G	S	**L**				Influenza JAP HA 529–537	i
	T	**Y**	V	S	V	S	T	S	**T**	**L**			Influenza A HA 210–219	k, l
	I	**Y**	S	T	V	A	S	S	**L**				Influenza A HA 518–526	k
	R	**Y**	L	E	N	G	K	E	**T**	**L**			HLA-A24 170–179	a, m
	R	**Y**	L	K	N	G	K	E	**T**	**L**			HLA-Cw3 170–179	a, m
	S	**Y**	I	P	S	A	E	K	**I**				P. berghei CSP 252–260	n
	S	**Y**	V	P	S	A	E	Q	**I**				P. yoelii CSP 281–289	o
	S	**Y**	I	G	S	I	N	N	**I**				RSV M2 82–90	p
	D	**Y**	A	T	L	G	V	G	**V**				HSV-1 ICP27 448–456	q
	L	**Y**	R	T	F	A	G	N	P	R	A		HSV-1 ICP27 322–332	q
	T	**Y**	K	D	T	V	Q	**L**					Polio VP1 111–118	r
	F	**Y**	D	G	F	S	K	V	P	**L**			Polio VP1 208–217	r
	A	**Y**	I	S	S	G	S	S	T	**L**			Human Ig VH 49–58	l
	N	**Y**	D	N	A	G	T	N	**L**				P. falciparum CSP 39–47	s
	K	**Y**	L	K	K	I	K	N	S	**L**			P. falciparum CSP 333–342	s
	K	**Y**	L	K	I	K	H	L	**L**				APC frameshift	t

* Also a T-cell epitope

References:
a: Falk et al. 1991 b; b: Rötzschke et al. 1990; c: Falk et al. 1991 a; d: Harpur et al. 1993; e: Sibille et al. 1990; f: Wallny et al. 1992; g: Pamer et al. 1991; h: Pamer 1994; i: Braciale et al. 1987; k: Kuwano et al. 1988; l: Cao et al. 1994; m: Maryanski et al. 1986; n: Romero et al. 1989; o: Weiss et al. 1990; p: Kulkarni et al. 1993; q: Banks et al. 1993; r: Kutubuddin et al. 1992; s: Blum-Tirouvanziam et al. 1994; t: Townsend et al. 1994; u: Reich et al. 1994

Table 1 (Continued)
B H-2D^d

	Position										Source	Ref.
	1	2	3	4	5	6	7	8	9			
Anchor or auxiliary anchor residues		**G**	**P**		R K				**I** **L** **F**			a, b
Other preferred residues				D E Q		N I L	D E					
Examples for ligands	K	**G**	**P**	I	T	V	Q	**I**			Unknown	b
	V	**G**	**P**	Q	K	N	E	N	**L**		Unknown	b
	S	**G**	**P**	R	K	X	I	X	**L**		Homol. mRNA CD40 mouse	b
	A	**G**	**P**	D	R	T	E	K	X	**L**	Unknown	b
	K	**G**	**P**	D	K	G	N	E	**F**		Homol. metalloproteinase 2 inhibitor	b
	I	**G**	**P**	E	R	G	H	N	**L**		Homol. hypoxanthine phosphoribosyl-transferase	b
	D	**G**	**P**	V	R	E	H	N	**L**		Homol. urease canavalia ensiformis	b
	K	**G**	**P**	E	R	X	N	G	**L**		Unknown	b
	S	**G**	**P**	E	R	G	E	K	**L**		Homol. proliferating cell nucleolar antigen P40	b
	D	**G**	**P**	V	R	G	I	S	**I**		Homol. ribosomal protein S17 rat	b
	N	**G**	**P**	Q	R	I	Y	N	**L**		Unknown	b
	S	**G**	**P**	V	A	L	V	N	F	**I**	Unknown	b
	I	**G**	**P**	N	R	A	F	N	**F**		Unknown	b
	S	G	**P**	E	R	**L**	L	S	X	Y	Homol. heterog. nucl. RNP complex K	b
	V	**G**	**P**	S	G	K	Y	F	I	**L**	Unknown	b
	F	**G**	**P**	Y	K	L	N	R	**L**		Homol. feline leukemia virus envelope polyprotein	b
	F	**G**	**P**	L	K	F	N	V	L	T	Unknown	b
	A	**G**	**P**	D	R	F	I	X	X	M	Unknown	b
	F	**G**	**P**	Y	R	F	Y	V	L	T	Unknown	b
	S	E	Q	D	L	N	F				Unknown	b
	S	X	H	K	E	Q	P	A	T		Homol. transforming protein spi-1 human	b
	S	X	**P**	K	T	D	X	Q	T	**L**	Homol. insulin receptor precursor	b
T-cell epitopes		**G**	**P**	P	H	S	N	N	F	G	Y Tum-P35B 4–13	c
	R	**G**	**P**	G	R	A	F	V	T	**I**	HIV gp160 318–327	d, f
	L	M	G	Y	I	P	L	V	G	A	HCV core 133–142	e

References:
a: Falk and co-workers, unpublished; b: Corr et al. 1993; c: Szikora et al. 1993; d: Takahashi et al. 1988; e: Shirai et al. 1994; f: Bergmann et al. 1993b

Table 1 (Continued)
C H-2L^d

	Position										Source	Ref.
	1	2	3	4	5	6	7	8	9			
Anchor residues		**P** **S**							**F** **L** **M**			a, b, c
Other preferred residues			G Q M L	T	T	I K F	F	Q N				
Examples for ligands	Y	**P**	H	F	M	P	T	N	**L***		MCMV pp 89 168–176	d
	L	**S**	P	F	P	F	D	**L***			OGDH 105–112	e
V A I T R I E Q	L	**S**	P	F	P	F	D	**L***			OGDH 97–112	e
	X	**P**	L	E	A	N	Y	Q	X	**F**	Unknown	c
	A	**P**	Q	P	G	M	E	N	**F**		Unknown	c
	Q	**P**	Q	R	G	R	E	N	**F**		Unknown	c
	X	**P**	Q	P	G	R	E	Q			Unknown	c
	X	**P**	Q	P	N	L	Y	Q	**L**		Unknown	c
	X	**P**	A	X	A	Y	P	Y			Unknown	c
	Y	**P**	N	V	N	I	H	N	**F**		Unknown	c
	X	**P**	Q	K	A	G	G	F	L	**M**	Phosphoglycerate kinase 180–189	c
T-cell epitopes	R	**P**	Q	A	S	G	V	Y	**M**		LCMV NP 118–126	f, g
	I	**S**	T	Q	N	H	R	A	**L**		Tumor antigen P91A 12–20	h
	L	**P**	Y	L	G	W	L	V	**F**		Tumor antigen P815 35–43	i
	A	**P**	T	A	G	A	F	F	**F**		JHMV Nucleocapsid 318–326	k
	Y	**P**	A	L	G	L	H	E	**F**		Measles NP 281–289	l
	T	**P**	H	P	A	R	I	G	**L**		E. coli β-gal. 876–884	m
	D	**P**	V	I	D	R	L	Y	**L**		Measles HA 343–351	n
	S	**P**	G	R	S	F	S	Y	**F**		Measles HA 544–552	n

* Also a T-cell epitope

References:
a: Falk et al. 1991b; b: Falk and co-workers, unpublished; c: Corr et al. 1992; d: Reddehase et al. 1989; e: Udaka et al. 1992; Udaka et al. 1993; f: Whitton et al. 1989; g: Schulz et al. 1991; h: Lurquin et al. 1989; i: Lethé et al. 1992; k: Bergmann et al. 1993a; l: Beauverger et al. 1993; m: Gavin et al. 1994; n: Beauverger et al. 1994

Table 1 (Continued)
D H-2K^b

	Position									Source	Ref.
	1	2	3	4	5	6	7	8			
Anchor or auxiliary anchor residues			Y		**F** **Y**			**L** **M** I V			a
Other preferred residues	R I L S A	N	P	R D E K T		T I E S	N Q K				
Examples for ligands	R	G	Y	V	**Y**	Q	G	**L***		VSV NP 52–59	b
	S	I	I	N	**F**	E	K	**L***		Ovalbumin 258–276	a, c, d
	H	I	Y	E	**F**	P	Q	**L**		Unknown	n
T-cell epitopes	I	I	Y	R	**F**	L	L	**I**		Rotavirus VP7 33–40	e
	S	S	I	E	**F**	A	R	**L**		HSV glycoprotein B 498–505	f
F	A	P	G	N	**Y**	P	A	**L**		Sendai virus NP 324–332	g, h
	K	S	P	W	**F**	T	T	**L**		MuLV p15E 574–581	i, k
	V	G	P	V	**F**	P	P	G	**M**	Rotavirus VP6 376–384	l
Y	S	G	Y	I	**F**	R	D	**L**		Rotavirus VP3 585–593	l
	F	E	Q	N	T	A	Q	A+		MUT 2 tumor antigen	m
	F	E	Q	N	T	A	Q	P+		MUT 1 tumor antigen	m

* Also a T-cell epitope
+ One of these peptides was found in a total cell extract of K^b-expressing tumor cells

References:
a: Falk et al. 1991b; b: van Bleek and Nathenson 1990; c: Rötzschke et al. 1991; d: Carbone et al. 1988; e: Franco et al. 1993; f: Bonneau et al. 1993; g: Kast et al. 1991; h: Schumacher et al. 1991; i: Sijts et al. 1994; k: White et al. 1994; l: Franco et al. 1994; m: Mandelboim et al. 1994; n: Wallny 1992

Table 1 (Continued)
E H-2D^b

	Position											Source	Ref.
	1	2	3	4	5	6	7	8	9				
Anchor residues					**N**				**M** **I**				a
Preferred residues		M	I L P V	K E Q V		L F							
Others	A N I F P S T V	A Q D	G	D T		A Y T V M E Q H I K P S	D E Q V T Y	F H K S Y					
Examples for ligands	A	S	N	E	**N**	M	E	T	**M***			Influenza A34 NP 366–374	a, b, c
	I	Q	V	G	**N**	T	R	T	**I***			Yersinia YOP 51 249–257	n
T-cell epitopes	A	S	N	E	**N**	M	D	A	**M**			Influenza A68 NP 366–374	d
	S	A	I	N	**N**	Y	A	Q	K	**L**		SV 40 T 206–215	e, o
	C	K	G	V	**N**	K	E	Y	**L**			SV 40 T 223–231	e, o
	Q	G	I	N	**N**	L	D	N	**L**			SV 40 T 489–497	e, o
	S	G	P	S	**N**	T	P	P	E	**I**		Adenovirus 5 E1A 234–243	f
	F	Q	P	Q	**N**	G	Q	F	**I**			LCMV NP 396–404	g
	S	G	V	E	**N**	P	G	G	Y	C	**L**	LCMV GP 276–286	h
	K	A	V	Y	**N**	F	A	T	C	G		LCMV GP 33–42	i, k
	R	A	H	Y	**N**	I	V	T	F			HPV16 E7 49–57	l
	N	N	L	D	**N**	L	R	D	Y	**(L)**		SV 40 T 492–500 (501)	m

* Also a T-cell epitope

References:
a: Falk et al. 1991b; b: Rötzschke et al. 1990; c: Townsend et al. 1986; d: Cerundolo et al. 1991; e: Deckhut et al. 1992; f: Kast et al. 1989; g: Yanagi et al. 1992; h: Oldstone et al. 1988; i: Oldstone et al. 1993; k: Klavinskis et al. 1990; l: Feltkamp et al. 1993; m: Alsheikly 1994; n: Starnbach and Bevan 1994; o: Tevethia et al. 1990

Table 1 (Continued)
F H-2K^k

	Position									Comments	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor residues		**E**						**I**	**I**	C-terminus at P8 or P9	a, b, c
Preferred residues	V F	D	K N Y M Q I L F P H T	L	A G P T V F S	N K H	T				
										Source	
Examples for natural ligands	H	**E**	T	T	F	N	S	**I**		β Actin 275–282	k
	D	**D**	H	R	A	G	K	**I**		S24 ribosomal protein 53–60	k
	Y	**E**	D	T	G	K	T	**I**		Unknown	k
	K	**E**	M	K	A	K	V	**I**		Homol. T cell transcript. factor 1	k
	E	**E**	E	P	V	K	K	**I**		Hn RNP C protein 84–91	k
	S	**E**	I	V	G	K	R	**I**		S7/S8 ribos. protein 137–144	k
	S	**E**	G	G	S	H	T	**I**		H-2D^k 112–119	k
	D	**E**	R	T	V	R	K	**I**		Unknown	k
	E	**E**	D	P	V	K	K	**V**		CArG bind. factor A 209–216	k
	E	A	Y	L	G	K	K	**V**		BiP 158–165	k
T-cell epitopes	F	**E**	A	N	G	N	L	**I**		Influenza A HA 259–266	c, i
	I	**E**	G	G	W	T	G	M	**I**	Influenza A HA 10–18	c, i
	S	**D**	Y	E	G	R	L	**I**		Influenza A NP 50–57	d, l
	F	**E**	S	T	G	N	L	**I**		Influenza JAP HA 255–262	e
	S	**E**	F	L	L	E	K	R	**I**	SV 40 T 560–568	f
	Y	**E**	N	D	I	E	K	K	**I**	P. falciparum CSP 375–383	g
	D	**E**	L	D	Y	E	N	D	**I**	P. falciparum CSP 371–379	g
	T	**E**	M	E	K	E	G	K	**I**	HIV-1 RT 206–214	h
	V	**E**	A	E	I	A	H	Q	**I**	Rabies NS 197–205	i
	E	**E**	G	A	I	V	G	E	**I**	Influenza A NSI 152–160	a

References:
a: Cossins et al. 1993; b: Norda et al. 1993; c: Gould et al. 1991; d: Bástin et al. 1987; e: Sweetser et al. 1989; f: Rawie et al. 1988; g: Kumar et al. 1988; h: Hosmalin et al. 1990; i: Larson et al. 1991; Gould et al. 1987; k: Brown et al. 1994; l: Gould et al. 1989

G H-2K^{km1}

	Position								Source	Ref.
	1	2	3	4	5	6	7	8		
Anchor or auxiliary anchor residues		**E**						**I**		a
Other preferred residues		Q G P	K N Q G M P Y	P	A R K		R Y			

References:
a: Norda et al. 1993

Table 1 (Continued)
H Qa-2

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		M L Q	N I L		V I	K M I	**H**		**L** **I** **F**		a, b
Other preferred residues	K A E Q		T	P E A G K S D	L T E H M F Y	L F N Y	R	E Q N D K S T R			
Examples for ligands	K	Q	N	P	I	A	**H**	Q	**L**	Unknown	b
	A	L	A	E	L	P	**H**	E	**L**	Unknown	b
	K	Q	N	P	T	V	**H**	H	**L**	Unknown	b
	A	G	L	L	G	M	**R**	S	G **L**	Unknown	b
	K	L	I	K	V	Y	**H**	S	**L**	Unknown	b
	V	Q	N	X	T	M	**H**	P	**I**	Unknown	b
	R	S	N	G	Q	V	**H**	M	**L**	Unknown	b
	K	L	T	G	I	K	**H**	E	**L**	Cofilin 127–135	b
	A	M	L	A	T	Y	**H**	K	**L**	Unknown	b
	G	Q	L	X	V	X	**H**	K	**L**	Unknown	b
	I	L	M	E	H	I	**H**	K	**L**	Ribosomal protein L19 137–145	b
	A	Q	N	P	V	L	Y	Q	**I**	Unknown	b
	S	L	I	K	T	V	**R**	E	**M**	Unknown	b
	K	M	L	P	V	E	**H**	N	**L**	Unknown	b
	K	Q	L	I	V	T	Y	H	**L**	Unknown	b
	S	V	L	D	D	L	A	L	**L**	Unknown	b
	Y	E	S	P	L	M	X	F	**L**	Unknown	b
	D	L	L	G	T	L	**H**	N	**L**	Unknown	b

References:
a: Rötzschke et al. 1993; b: Joyce et al. 1994

I Selected other T-cell epitopes

MHC	Sequence												Comments	Ref.
H-2D^k		R	R	K	G	K	Y	T	G	L			T cell epitope of LEC-A	a
H-2M3	fM		F	F	I	N	I	L	T	L	L	V	P	ND1α 1–17	b
	fM		F	F	I	N	A	L	T	L	L	V	P	ND1β 1–17	b

References:
a: de Bergeyck et al. 1994; b: Fischer Lindahl 1991

Table 2 HLA-A motifs
A HLA-A1

	Position												Source	Ref
	1	2	3	4	5	6	7	8	9					
Anchor or auxiliary anchor residues		T S	**D** **E**	P			**L**		**Y**					a, b, c, f, i
Other preferred residues		L		G I	G N Y	G V I								
Examples for ligands	A	T	**D**	F	K	F	A	M	**Y**				Cyclin-like protein 135–143	a, i
	I	A	**D**	M	G	H	L	K	**Y**				Proliferation cell nuclear antigen 241–249	a, b, i
	M	I	**E**	P	R	T	L	Q	**Y**				Ribosomal protein S16 40–48	a, b
	Y	T	S	D	Y	F	I	S	**Y**				Ets-1 154–162	a, i
	L	T	**D**	P	G	V	L	D	**Y**				Unknown	a
	V	S	**D**	I	V	G	P	D	G	L	V	**Y**	Fibrillarin 177–188	a, b
	Y	T	**D**	Y	G	G	L	I	F	N	S	**Y**	Cytochrome C oxidase II	a, i
	Q	S	**E**	D	G	S	H	T	I	Q	I	M **Y**	HLA class I α chain 111–123	a
	Y	L	**D**	D	P	D	L	K	**Y**				Cytosine methyl transferase 238–246	i
	S	T	**D**	H	I	P	I	L	**Y**				Fructose-6-amino transferase 217–225	i
	D	S	**D**	G	S	F	F	L	**Y**				IgG4 279–287	i
	G	T	**D**	E	X	R	N	X	**Y**				Unknown	i
	V	S	**D**	P	Y	N	X	K	**Y**				Unknown	d, i
	V	A	**D**	K	V	H	X	M	**Y**				Unknown	i
	Y	T	A	V	V	P	L	V	**Y**				J-chain 102–110	i
	Y	T	N	P	Q	F	N	V	**Y**				Unknown	i
	E	T	X	X	P	D	W	S	**Y**				Unknown	i
	F	T	**D**	V	N	S	X	X	R	**Y**			Unknown	i
	S	S	**E**	Q	T	F	M	Y	**Y**				Ornithine decarboxylase 309–317	b
	S	T	**E**	P	V	N	I	L	**Y**				Unknown	b
	G	T	**D**	P	G	V	L	I	**Y**				Unknown	b
	S	T	**E**	P	P	M	L	N	**Y**				Unknown	b
	S	L	**E**	P	Q	R	T	Q	**Y**				Unknown	b
	F	T	**E**	V	S	I	R	K	**Y**				Unknown	b
	K	F	**D**	P	V	N	L	V	**Y**				Unknown	b
	A	V	**D**	P	G	G	M	Y	S				Unknown	b
	F	G	S	G	A	R	D	X	**Y**				Unknown	b
	Y	X	**E**	P	Q	F	L	T	**Y**				Unknown	b
	A	X	I	P	A	F	I	N	**Y**				Unknown	b
	I	T	**E**	D	M	G	H	L	K	**Y**			Unknown	f
	E	T	**D**	X	X	X	D	R	S	E	**Y**		Unknown	i
T-cell epitopes	E	A	**D**	P	T	G	H	S	**Y**				MAGE-1 161–169	e, k
	V	S	**D**	G	G	P	N	L	**Y**				Influenza A PB1 591–599	b, f
	C	T	**E**	L	K	L	S	D	**Y**				Influenza A NP 44–52	f
	E	V	**D**	P	I	G	H	L	**Y**				MAGE-3	g, h

References:
a: Falk et al. 1994c; b: Di Brino et al. 1993b; c: Sette et al. 1994; d: Engelhard 1994; e: Traversari et al. 1992; f: DiBrino et al. 1994; g: Gaugler et al. 1994; h: Celis et al. 1994; i: Kubo et al. 1994; k: Van der Bruggen et al. 1991

Table 2 (Continued)
B HLA-A*0201

	Position															Source	Ref.
	1	2	3	4	5	6	7	8	9								
Anchor or auxiliary anchor residues		L M				V			V L								a
Preferred residues				E K				K									
Other residues	I L F K M Y V		A Y F P M S R	G P D T	I K Y N G F V H	I L T	A Y H	E S									
Examples for ligands	S	L	L	P	A	I	V	E	L							Protein phosphatase 2A 389–397	b
	Y	L	L	P	A	I	V	H	I							ATP-dependent RNA Helicase 148–156	b
	T	L	W	V	D	P	Y	E	V							B-cell transloc. gene 1 protein 103–111	b
	S	X	P	S	G	G	X	G	V							Unknown	b
	G	X	V	P	F	X	V	S	V							Unknown	b
	S	X	X	V	R	A	X	E	V							Unknown	b
	K	X	N	E	P	V	X	X	X							Unknown	b
	A	L	W	G	F	F	P	V	X+							Unknown mouse protein	b, e
	L	L	D	V	P	T	A	A	V							IP-30 signal sequence 27–35	b
L	L	L	D	V	P	T	A	A	V							IP-30 signal sequence 26–35	b, c
L	L	L	D	V	P	T	A	A	V	Q	A					IP-30 signal sequence 26–37	b, c
L	L	L	D	V	P	T	A	A	V	Q						IP-30 signal sequence 26–36	c
	V	L	F	R	G	G	P	R	G	L	L	A	V	A		SSR α signal sequence 12–25	d, c
	M	V	D	G	T	L	L	L	L							HLA-E signal sequence 1–9	b
	Y	M	N	G	T	M	S	Q	V+							Tyrosinase 369–377	f, g, h, i
	M	L	L	S	V	P	L	L	L	G						Calreticulin signal sequence 1–10	c
	S	L	L	G	L	L	V	E	V*							Unknown	l
	A	L	L	P	P	I	N	I	L*							Unknown	l
	T	L	I	K	I	Q	H	T	L*							Unknown	l
	A	L	I	V	G	X	N	D	D*							Unknown	l
	H	L	I	D	Y	L	V	T	S*							Carboxypeptidase M 91–99	l
	I	L	A	P	P	V	V	K	L	L	V*					Unknown	l
	A	L	F	P	Q	L	V	I	L*							Unknown	l
	G	I	L	G	F	V	F	T	L+							Influenza matrix protein 58–66	a, k, o, y, z
T-cell epitopes	I	L	K	E	P	V	H	G	V							HIV-1 RT 476–484	a, e, j
	I	L	G	F	V	F	T	L	T	V						Influenza matrix protein 59–68	a, k
	L	L	F	G	Y	P	V	Y	V							HTLV-1 tax 11–19	o
	G	L	S	P	T	V	W	L	S	V						Hepatitis B sAg 348–357	m
	W	L	S	L	L	V	P	F	V							Hepatitis B sAg 335–343	m
	F	L	P	S	D	F	F	P	S	V						Hepatitis B Nucleocapsid 18–27	n
	C	L	G	G	L	L	T	M	V							EBV LMP2 426–434	p
	F	I	A	G	N	S	A	Y	E	Y	V					HCMV glycoprotein B 618–628	m
	K	L	G	E	F	Y	N	Q	M	M						Influenza B NP 85–94	q
	K	L	V	A	L	G	I	N	A	V						HCV-1 1406–1415	r
	D	L	M	G	Y	I	P	L	V							HCV core 132–140	s
	R	L	V	T	L	K	D	I	V							HPV 11 E7 4–12	t
	M	L	L	A	L	L	Y	C	L							Tyrosinase 1–9	f, g
	A	A	G	I	G	I	L	T	V							Melan A/Mart 1	w, x
	Y	L	E	P	G	P	V	T	A							pmel 17/gp100	u
	I	L	D	G	T	A	T	L	R	L						pmel 17/gp100	v

* Class I ligands allocated to A2 by motif. + Also a T-cell epitope

References:
a: Falk et al. 1991b; b: Hunt et al. 1992; c: Henderson et al. 1992; d: Wei and Cresswell 1992; e: Henderson et al. 1993; f: Wölfel et al. 1994; g: Robbins et al. 1994; h: Brichard et al. 1993; i: Engelhard et al. 1993; j: Walker et al. 1989; k: Gotch et al. 1988; l: Harris et al. 1993; m: Nayersina et al. 1993; n: Bertoletti et al. 1993, 1994; o: Utz et al. 1992; p: Lee et al. 1993; q: Robbins et al. 1989; r: Chisari and co-workers, personal comm.; s: Shirai et al. 1994; t: Tarpey et al. 1994; u: Cox et al. 1994; v: Kawakami et al. 1994b; w: Coulie et al. 1994; x: Kawakami et al. 1994a, c; y: Falk et al. 1994a; z: Bednarek et al. 1991

Table 2 (Continued)
C HLA-A*0205

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		V L I M				I V L A			**L**		a
Other preferred residues		Q	Y P F I	G E D K N	Y V L I	T	Q	K			

References:
a: Rötzschke et al. 1992

D HLA-A3

	Position										Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**	**10**		
Anchor or auxiliary anchor residues		**L** **V** **M**	F Y			I M F V L	I L M F		**K** **Y** **F**	K		a, b, g
Other preferred residues	I				I P V K	T		Q S T K				
Examples for ligands	K	X	F	K	M	I	L	R	**K**		Unknown	a
	K	**L**	F	K	N	I	L	Y	**K**		Unknown	a
	Y	**L**	X	V	R	X	A	X	i	V	Unknown	a
	K	**L**	H	K	Q	R	A	K	S		Unknown	a
	S	**L**	F	K	Q	V	V	T	**K**		Unknown	a
	K	X	F	V	K	X	L	X	**Y**		Unknown	a
	S	**L**	F	N	T	H	L	X	**K**		Unknown	a
	T	**L**	A	N	D	X	V	V	P		Unknown	a
	G	**I**	F	A	X	X	X	V	K	A	Unknown	a
	T	X	F	V	K	X	L	X	**Y**		Unknown	a
	S	**L**	F	D	H	I	L	X	K	H	Unknown	a
	K	**L**	F	K	V	V	X	N	**Y**		Unknown	a
	K	**L**	Y	E	K	V	Y	T	Y	**K**	Unknown	a
	K	**L**	F	N	I	M	V	T	**Y**		Unknown	a
	K	**L**	F	E	K	V	Y	N	**Y**		Unknown	a
	K	**L**	F	K	V	T	F	S	**Y**		Unknown	a
	G	**L**	F	P	X	Q	F	A	**Y**		Unknown	a
	S	**L**	F	E	L	V	F	X	**Y**		Unknown	a
	S	**L**	X	E	K	T	F	D	**Y**		Unknown	a
	S	**L**	H	K	Y	X	f	e	**Y**		Unknown	a
	K	**M**	F	N	I	T	v	T	**Y**		Unknown	a
	K	**L**	F	V	K	V	y	N	**Y**		Unknown	a
	K	**I**	V	R	K	P	G	M	A		Unknown	a
T-cell epitopes	R	**L**	R	D	L	L	L	I	V	T	**R**	HIV-1 env gp41 768–778	c
	Q	**V**	P	L	R	P	M	T	Y	**K**		HIV-1 nef 73–82	d
	T	**V**	Y	Y	G	V	P	V	W	**K**		HIV-1 env gp120 36–45	e
	R	**L**	R	P	G	G	K	K	K			HIV-1 gag p17 20–29	e
	I	L	R	G	S	V	A	H	**K**			Influenza NP 265–273	f

References:
a: DiBrino et al. 1993a; b: Maier et al. 1994; c: Takahashi et al. 1991; d: Koenig et al. 1990; e: Venet and Walker 1993; f: DiBrino et al. 1993b; g: Kubo et al. 1994

Table 2 (Continued)
E HLA-A*1101

	Position											Source	Ref.
	1	2	3	4	5	6	7	8	9	10	11		
Anchor or auxiliary anchor residues		V I F Y	M L F Y I A				L I Y V F		**K**	**K**	K		a, b, c
Other preferred residues	A	T	N D E Q	P G D E K	P I F V M	I V M		R K N E Q	R D	R	R		
Examples for ligands	A	V	M	K	P	E	A	E	K	R	**K**	Unknown	b
	A	V	I	L	P	P	L	S	P	Y	F	**K** HSB 66 EST 18–29	b
	A	S	F	D	K	A	K	L	K	**K**		Thymosin β-10 11–20	b
	G	Q	Y	G	N	P	L	N	**K**			Cattle metalloproteinase 19–27	b
	G	V	M	P	S	H	F	S	**R**			Ribosomal protein S19 93–101	b
	Y	F	D	P	A	N	G	K	F	S	**K**	Elongation factor 2 265–275	b
	A	T	A	G	D	G	L	I	E	L	R **K**	Prohibitin (rat) 229–240	b, c
	S	T	Y	Y	G	S	F	V	T	**R**		Unknown (also presented by A33)	a, b
	S	V	L	N	L	V	I	V	**K**			Ribosomal protein S6 107–115	c
	K	V	V	N	P	L	F	E	**K**			Ribosomal protein L7A 25–33	c
	R	T	Q	N	V	L	G	E	**K**			Ribosomal protein S3 54–62	c
	G	T	M	T	T	S	X	Y	**K**			Unknown	c
	A	S	F	D	K	A	K	L	**K**			Thymosin β-10 11–19	c
	A	A	M	X	D	T	V	V	F	**K**		Unknown	c
	R	V	E	Q	A	V	E	S	M	V	**K**	Unknown	c
T-cell epitope	I	V	T	D	F	S	V	I	**K**			EBNA 4 416–424	a, d

References:
a: Zhang et al. 1993; b: Falk et al. 1994c; c: Kubo et al. 1994; d: Gavioli et al. 1993

F HLA-A24

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		**Y**			I V	F			**I** **L** **F**		a
Other preferred residues			N E L M P G	D P			Q N	E K			
Examples for ligands	K	**Y**	P	E	N	F	F	L	**L**	Protein phosphatase 1 113–121	b
	Y	**Y**	E	E	Q	H	P	E	**L**	NK/T-cell activation protein 107–115	b
	A	**Y**	V	H	M	V	T	H	**F**	Unknown	b
	V	**Y**	X	K	H	P	V	S	X	Unknown	b
T-cell epitope	R	**Y**	L	K	D	Q	Q	L	**L**	HIV gp 41 583–591	c

References:
a: Maier et al. 1994; b: Kubo et al. 1994; c: Dai et al. 1992

Table 2 (Continued)
G HLA-A*3101

	Position											Comments	Ref.
	1	2	3	4	5	6	7	8	9				
Anchor or auxiliary anchor residues		L V Y F	F L Y W			L F V I			**R**				a
Other preferred residues	K R	T Q	K N	P D E G S V T	P I V F L Y W	T N D E R	N V R F T H L Y	L R N Q				P1 deduced from individual ligands	
												Source	
Examples for ligands	L	Q	F	P	V	G	R	V	H	**R**		Histon H2 a 23–32	a
	Q	Q	L	Y	W	S	H	P	**R**			Ribosomal protein S29 (rat) 3–11	a
	R	G	Y	R	P	R	F	R	**R**			CCAAT-binding transcription factor 240–248	a
	K	V	F	G	P	I	H	E	**R**			[GlcNac]-P-transferase 371–379	a
	K	I	M	K	W	N	Y	E	**R**			Unknown	a
	R	Y	M	D	A	W	N	T	Y	S	**R**	Lamin B2	a
T-cell epitope	S	T	L	P	E	T	T	V	V	R	**R**	Hepatitis B cAg 141–151	b

References:
a: Falk et al. 1994c; b: Missale et al. 1993

H HLA-A*3302

	Position											Comments	Ref.
	1	2	3	4	5	6	7	8	9				
Anchor or auxiliary anchor residues		A I L F Y V							**R**				a
Preferred residues	D E	T	L K	P	P	I L F						P1 deduced from individual ligands	
Other possible residues	M		Q W E N	R D E G S H P	R I F P V L W	R D H Y	H Y V T S	Q N E M					
												Source	
Examples for ligands	D	M	A	A	Q	I	T	Q	**R**			HLA class I α-chain 161–169	a
	E	S	G	P	S	I	V	H	**R**			Actin 364–372	a
	T	Y	Y	G	S	F	V	T	**R**			Unknown	a
	D	Y	I	H	I	R	I	Q	Q	**R**		Human cDNA HSB15F102 65–74	a
	E	I	M	K	W	N	R	E	**R**			Unknown	a
	T	I	M	P	K	D	I	Q	L	A	R	**R**	Histon 3.1/3.3 118–129	a
T-cell epitope	I	V	G	L	N	K	I	V	**R**			HIV p24 gag 267–275	b, c

References:
a: Falk et al. 1994c; b: Buseyne et al. 1993; c: Buseyne and Riviere 1993

Table 2 (Continued)
I HLA-A68.1

	Position											Source	Ref.
	1	2	3	4	5	6	7	8	9				
Anchor residues		**V**							**R**				a
		T							**K**				
Examples for ligands	A	**V**	A	A	V	A	A	R	**R**			Unknown	a
	E	**V**	A	P	P	E	Y	H	**R**			Unknown	a
	E	**V**	A	P	P	E	Y	H	R	**K**		Unknown	a
	D	**V**	F	R	D	P	A	L	**K**			Homologous ribosomal 60S	a
	K	**T**	G	G	P	I	Y	K	**R**+			Influenza NP 91–99	a, b
	E	**V**	I	L	I	D	P	F	H	**K**		Unknown	a
	T	**V**	F	D	A	K	R	L	I	G	**R**	HSP 70B / HSC70 66–76	a
	X	**V**	L	K	X	I	A	K	**R***			Unknown	d
	P	**V**	K	Q	V	V	Y	H	**R***			Unknown	d
	E	S	G	P	S	I	V	H	R	**K***		β-Actin 364–373	d
	T	**T**	X	T	T	T	N	A	**R***			Unknown	d
	D	**T**	T	P	T	X	X	**R***				Unknown	d
T-cell epitopes	S	**T**	L	P	E	T	T	V	V	R	**R**	Hepatitis B cAg 141–151	c

* Class I ligands allocated to A68.1 by motif +Also a T-cell epitope

References:
a: Guo et al. 1992; b: Silver et al. 1992; c: Missale et al. 1993; d: Harris et al. 1993

Table 3 HLA-B motifs
A HLA-B7

	Position											Source	Ref.
	1	2	3	4	5	6	7	8	9				
Anchor or auxiliary anchor residues		**P**	R						**L**				a, b
									F				
Other preferred residues				D	D	F	L						
				G	P	T							
Also detected	A		D	E	I	R	V						
	H		E	H	V	L							
	S		Q	L		I							
			K	K									
			Y	S									
			F	T									
			M	P									
			N										
			A										
Examples for ligands	A	**P**	R	T	V	A	L	T	A			HLA-DP signal sequence 9–17	a
	A	**P**	R	T	V	A	L	T	A	**L**		HLA-DP signal sequence 9–18	a
	A	**P**	R	A	X	X	X	X	X			Unknown	a
	A	**P**	R	X	P	X	T	G	X			Unknown	a
	A	**P**	R	A	S	R	P	S	X			Unknown	a
	A	**P**	R	T	L	V	L	L	**L**			HLA-A2.1 signal sequence 5–13	a
	M	**P**	R	G	V	V	V	T	X			Unknown	a
	S	**P**	R	Y	I	F	T	M	**L**			Topoisomerase II 801–809	a
	A	**P**	A	P	T	V	A	V	X			Unknown	a
	R	**P**	S	G	P	G	P	E	X			Unknown	a
	L	V	M	A	P	R	T	V	**L**			HLA-B7 signal sequence 2–10	a
	R	V	M	A	P	R	A	X	X			Unknown	a
	A	**P**	R	A	F	X	P	X	P	V		Unknown	a
	A	A	S	K	E	R	S	G	V	S	**L**	Histone H1 49–59	a
	A	**P**	R	S	N	G	M	V	X			Unknown	c
	A	**P**	R	Q	P	G	X	M	A			Unknown	c
	A	**P**	A	P	P	P	K	p	M			Ribosomal S26 protein 107–115	c
	A	**P**	Y	G	G	P	X	A	X			Unknown	c
T-cell epitope	T	**P**	G	P	G	V	R	Y	P	**L**		HIV-1 nef 128–137	d

References:
a: Huczko et al. 1993; b: Maier et al. 1994; c: Engelhard 1994; d: Culmann et al. 1991

Table 3 (Continued)
B HLA-B8

	Position									Source	Ref.
	1	2	**3**	4	**5**	6	7	8	**9**		
Anchor residues			**K**		**K** **R**				**L**		a, b, d
Other preferred residues	G L I		R	E Q D H L S T R G K		N Q H I L Y V E M S T F	E H M N D Q S **T** Y	E Q H S L V D T	F M		
Examples for ligands	H	P	**K**	Y	**K**	T	E	**L**		Tristetraproline 148–155	d
	I	L	**K**	Q	**K**	I	A	d	**l**	IL-6 precursor 161–169	d
	E	L	**K**	V	**K**	N	l	e	**l**	Restin 1273–1281	d
	E	P	**K**	Y	**K**	T	Q	**L**		Yeast PRAI-SCS 95–102	d
	V	P	**K**	L	**K**	V	X	A	**L**	Rat ribosomal prot. L18, 94–102	d
	F	A	**K**	P	**R**	V	G	G		Unknown	d
	S	P	**K**	L	**K**	Y	M	Q		Unknown	d
	E	L	**K**	K	**K**	T	N	**l**		Unknown	d
	S	P	**K**	E	**K**	I	X	Y		Unknown	d
	X	A	**K**	E	**K**	L	A	D		Unknown	d
	S	P	**K**	E	**K**	Y	E	X		Unknown	d
	E	L	**K**	E	**K**	T	Q	**L**		Unknown	d
	L	P	**K**	V	**K**	**L**	A	**L**		Unknown	d
	H	p	**K**	Y	**K**	T	E	**L**		Unknown	d
	V	L	D	L	**K**	I	V	A	**F**	Unknown	d
	G	P	**K**	E	**K**	X	A	**M**		Unknown	d
	G	L	**K**	V	**K**	G	N	E	**F**	Unknown	d
	F	L	**K**	P	**K**	F	V	A	**L**	Unknown	d
	E	L	**K**	I	**K**	V	Y	X	**l**	Unknown	d
	S	L	**K**	E	**K**	V	X	**L**		Unknown	d
	E	L	**K**	E	**K**	X	y	e	**l**	Unknown	d
	I	P	**K**	L	**K**	N	V	K	r	Unknown	d
	S	L	**K**	I	**K**	X	L			Unknown	d
	D	L	**K**	Q	**K**	N	E	**L**		Unknown	d
T-cell epitopes	E	L	**R**	S	**R**	Y	W	A	**I**	Influenza NP 380–388	b
	F	L	**R**	G	**R**	A	Y	G	**L**	EBNA 3 339–347	c
	E	I	Y	K	**R**	W	I	I	**L**	HIV gag p24, 262–270	d, e
	G	E	I	Y	**K**	R	W	I	**I**	HIV gag p24, 261–269	d, e
	E	**I**	**K**	D	T	K	E	A	**L**	HIV gag p17, 93–101	d, f

References:
a: Malcherek et al. 1993; b: Sutton et al. 1993; c: Burrows et al. 1990; d: DiBrino et al. 1994; e: Phillips et al. 1989; f: Achour et al. 1990

Table 3 (Continued)
C HLA-B*2702

	Position										Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**			
Anchor residues		**R**							**F Y I L W**			a
Other preferred residues	K		**F L X**	G P K D E Q T S	I K E V M T	I V Y R D H E Q	Y L V T F	K V D E R				
Examples for ligands	S	**R**	D	K	**T**	I	I	M	**W**		HGNBPβ-subunit 35–43	a
	G	**R**	L	T	K	H	T	K	**F**		Rat ribosomal protein L36 36–44	a
	R	**R**	F	V	N	V	V	P	**T**	**F**	Human fau protein 114–123	a
	K	**R**	Y	K	S	I	V	K	**Y**		HFPS 191–199	a
	K	**R**	K	K	A	Y	A	D	**F**		Cytochrome C oxidase 42–50	a
	K	**R**	G	I	L	T	L	K	**Y**		Actin 63–71	a
	G	**R**	F	G	V	G	N	R	**Y**		Unknown	a
	G	**R**	F	K	L	I	V	L	**Y**		Unknown	a

References:
a: Rötzschke et al. 1994

Table 3 (Continued)
D HLA-B*2705

	Position												Source	Ref.
	1	2	3	4	5	6	7	8	9					
Anchor residues		**R**							**L** **F**					a, b
Other preferred residues	A G K R		L I F	K Q E G	I V L P G	I A N Q D V K	I T Y M L W N V P	K N R E Q	Y M I R H K					
Examples for ligands	G	**R**	L	T	K	H	T	K	**F**				Rat ribosomal protein L 36 36–44	b
	A	**R**	L	F	G	I	R	A	**K**				HBBCP 190–198	a, b
	R	**R**	L	G	X	Q	Y	R	**R**				Unknown	b
	R	**R**	F	G	D	K	L	N	**F**				Immediate early response gene 87–95	b
	G	**R**	v	F	I	I	K	E					Homologous to Il-1 receptor antagonist	b
	G	**R**	X	X	i	f	X	r	f	G	Y	**H**	Unknown	b
	T	**R**	Y	P	I	L	A	G	**H**				Cytochrome P450 20–28	b
	r	**r**	i	s	g	V	D	R	**Y**				Unknown	a, b
	K	**R**	F	S	F	K	K	S	**F**				Cattle MARCKS 155–163	b
	A	**R**	L	Q	T	A	L	L					Rat core histone 188–196	b
	R	**R**	X	P	I	F	S	R	**L**				TIS 11B protein 325–333 (X = L)	b
	R	**R**	F	M	q	Y	Y	V	**Y**				Homologous to proteasome subunit C5 127–135	b
	R	**R**	I	K	E	I	V	K	K	**H**			HSP 86 200–209	b
	R	**R**	M	G	P	P	V	G	G	H	**R**		Ribonucleoprotein L 312–322	b
	R	**R**	I	K	E	I	V	K	**K**				HSP 89 a 200–208	a
	G	**R**	I	D	K	P	I	L	**K**				Ribosomal protein L8 173–181	a
	R	**R**	S	K	E	I	T	V	**R**				ATP-dependent RNA helicase 77–85	a
	K	**R**	F	E	G	L	T	Q	**R**				Unknown	a
	R	**R**	V	K	E	V	V	K	**k**				HSP 89 B 195–203	a
	F	**R**	Y	N	G	L	I	H	**R**				60 S ribosomal protein L28 37–45	a
	R	**R**	Y	Q	K	S	T	E	**L**				Histon H3.3 52–60	a
	R	**R**	W	L	P	A	G	d	a				Elongation factor 2 341–349	a
	R	**R**	I	S	G	V	D	R	**Y**				Unknown	a
	R	**R**	F	T	R	P	E	H					Unknown	a
	K	K	Y	Q	K	S	T	E	**L**				Unknown	c
	XXLNSQDQQCDSSLVE												Draft-1 protooncogene 1–16	i
B*270x-restricted T-cell epitopes	S	**R**	Y	W	A	I	R	T	**R**				Influenza NP 383–391	d
	R	**R**	R	W	R	R	L	T	**V**[x]				EBNA LMP2 236–244	e
	R	**R**	Y	P	D	A	V	Y	**L**				Measles F protein 438–446	f
	R	**R**	I	Y	D	L	I	E	**L**				EBNA 3C 258–266	e
	K	**R**	W	I	I	L	G	L	N	**K**			HIV-1 gag p24 265–274	d, g
	G	**R**	A	F	V	T	I	G	**K**				HIV-1 gp120 314–322	d
	R	**R**	K	A	M	F	E	D	**I**				HSP 60 284–292	h

[x] B*2704-restricted

References:
a: Jardetzky et al. 1991; b: Rötzschke et al. 1994; c: Shepherd et al. 1993; d: Huet et al. 1990; e: Brooks et al. 1993; f: van Binnendijk et al. 1993; g: Buseyne et al. 1993; h: Cerrone et al. 1991; i: Frumento et al. 1993

Table 3 (Continued)
E HLA-B*3501

	Position										Source	Ref.
	1	2	3	4	5	6	7	8	9	10		
Anchor or auxiliary anchor residues		**P**							**Y** **F** **M** **L** **I**	**Y**		a, b
Other preferred residues	M	A V Y R D	I L F V M E T Y N	K D E G P	D I V T E G L M	I Q K V L M	V N E Q T K	E Q V T				
T-cell epitopes	K	**P**	K	D	E	L	D	**Y**			P. falciparum CSP 368–375	a
	K	S	K	D	E	L	D	**Y**			P. falciparum CSP 368–375	a
	K	**P**	N	D	K	S	L	**Y**			P. falciparum LS 1850–1857	a
	A	S	R	C	W	V	A	**M**			HCV E1 235–242	c

References:
a: Hill et al. 1992; b: Falk et al. 1993b; c: Koziel et al. 1992

F HLA-B*3701

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		**D** E			V I			**F** **M** **L**	**I** **L**		a
Other preferred residues	K Q	H P G S L			T R A D G H M		Q K Y L	T E N D Q G H			
T-cell epitope	E	**D**	L	R	V	L	S	**F**	**I**	Influenza NP 339–347	b

References:
a: Falk et al. 1993b; b: Townsend et al. 1986

G HLA-B*3801

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		**H**	**D** **E**						**F** **L**		a
Other preferred residues	I	F P W Y	I A S N M V	G E P L V	M T V A E G L K S	V I T K R N H	Y V N	K Y N R T	I		
Examples for ligands	E	H	A	G	V	I	S	V	**L**	Unknown	a
	T	H	D	E	L	E	D	K	**L**	Unknown	a
	Q	Y	D	E	A	V	A	Q	**F**	Histone binding protein 627–635	a
	Y	P	D	P	A	N	G	K	**F**	Elongation factor 2 265–273	a
	S	H	I	G	D	A	V	V		Cyclin 152–159	a
	Y	H	E	D	I	H	T	Y	**L**	Cyclin A 178–186	a
	T	F	D	V	A	P	S	R	**L**	Pm5 protein 270–278	a

References:
a: Falk et al. 1995b

H HLA-B*39011

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		**R** **H**				I V L			**L**		a
Other preferred residues			A D I L F V M S T Y	D E G P K	V Y I L F T G K N P	N	N Y F	S K R E T	V I M		
Examples for ligands	S	**H**	I	G	D	A	V	**V**		Cyclin 152–159	a
	I	**H**	E	P	E	P	H	**I**		CKShs1 protein 59–66	a
	S	**R**	D	K	T	I	I	**M**		GBLP 35–42	a

References:
a: Falk et al. 1995b

Table 3 (Continued)
I HLA-B*3902

	Position									Ref.
	1	**2**	3	4	5	6	7	8	**9**	
Anchor or auxiliary anchor residues		**K** **Q**			I L F V				**L**	a
Other preferred residues	K A		A I F V N L T Y E H S	G P	N E G P Q S T	V Y T H F I M P R	V L T Y N D H	T S R	F M	

References:
a: Falk et al. 1995b

K HLA-B40*

	Position											Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**	10	11		
Anchor or auxiliary anchor residues		**E**	F I V						**L** **W** **M** **A**				a
Examples for ligands	T	**E**	F	P	K	E	R	H	L	R	**L**	Unknown	a
	G	**E**	F	P	N	K	N	X	**L**			Unknown	a
	G	**E**	F	P	N	K	N	X	L	Y	**A**	Unknown	a
	G	**E**	F	P	G	K	I	F	L	Y	**A**	Unknown	a
	W	**E**	F	L	Q	P	I	L	**L**			Unknown	a
	G	**E**	F	I	P	G	N	D	L	H	R	Unknown	a
	G	**E**	F	P	P	X	D	N	**W**			Unknown	a
	E	**E**	F	Y	V	D	L	E	R			HLA-DQ α 33–41	a
	N	**E**	F	P	D	I	D	I	R			Unknown	a
	A	**E**	F	P	K	X	E	A	R			Unknown	a
	A	**E**	I	G	E	V	I	V	L	W	X **W**	Unknown	a
	A	**E**	I	P	G	E	I	A	**L**			Unknown	a
	G	**E**	I	L	D	V	F	D	**A**			IRE-BP 695–703	a
	F	**E**	I	P	X	L	D	V	**A**			Unknown	a
	D	**E**	V	T	P	Q	P	Q	L	V		Unknown	a
	K	**E**	V	G	V	D	V	A	L	Y	**A**	Unknown	a
	K	**E**	S	T	L	H	L	V	**L**			Ubiquitin 63–71	a
	G	**E**	V	D	V	E	Q	H	T			Cyclin B 313–321	a
	H	**E**	E	T	P	P	T	T	S			c-myc 241-249	a

* Motif and ligands deduced by exclusion: Class I ligands from a c-*myc* transfected B-cell line expressing A2, A68, and B40 were sequenced. Those not containing an A2 or A68 motif were thought to contain B40 ligands.

References:
a: Harris et al. 1993

Table 3 (Continued)
L HLA-B*4402

	Position										Ref.
	1	**2**	3	4	5	6	7	8	**9**	10	
Anchor or auxiliary anchor residues		**E**							**F** **Y**	F Y	a
Preferred residues	A S		M I L D		I	V	Y				
Others	D		N	P R K							

References:
a: Fleischhauer et al. 1994

M HLA-B*4403

	Position										Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**	10		
Anchor or auxiliary anchor residues		**E**							**Y** **F**	Y F		a
Preferred residues	A S		M I L V D									
Others			N	P R K	I V K		Y F					
Examples for ligands	A A	**E** **E**	D M	K G	E K	N G	Y S	K F	K K	**F** **Y**	HSP 90 427–436 Elongation factor 2 48–57	a a
B*440x-restricted T-cell epitope	E	**E**	N	L	L	D	F	V	R	**F**	EBNA 6 130–139	b

References:
a: Fleischhauer et al. 1994; b: Khanna et al. 1992

Table 3 (Continued)
N HLA-B*5101

	Position									Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**		
Anchor or auxiliary anchor residues		**A** **P** **G**							**F** **I**		a
Other preferred residues	I L V Y D	W F	I L M F W Y V E H D R N	G V I K E D	V T G A I S	N I L K Q	K Q R E	T	W M V L		
Examples for ligands	Y	**P**	F	K	P	P	K	**V**		UBC5, yeast 61–68	a
	D	**A**	H	I	Y	L	N	H	**I**	Thymidylate synthase 253–261	a
	T	**G**	Y	L	N	T	V	T	**V**	GBLP 192–200	a
	d	**A**	Y	A	L	N	H	T	**L**	Unknown	a
	l	**P**	P	E	V	N	R	Q	**L**	Unknown	a

References:
a: Falk et al. 1995a

O HLA-B*5102

	Position									Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**		
Anchor or auxiliary anchor residues		**P** **A** **G**	Y						**I** **V**		a
Other preferred residues			F V L I	G E K L T Q R N H	V Q N G T	I N Q T	R E Q K	T R Y			
Examples for ligands	Y	**A**	Y	D	G	K	D	Y	**I**	MHC I α chain 140–148	a
	Y	**P**	F	K	P	P	K	**V**		UBC5, yeast 61–68	a
	L	**P**	P	G	R	I	I	K	X	Unknown	a
	L	**P**	F	T	V	I	L	v		CDC25 homol. 560–567	a
	T	**G**	Y	L	N	T	V	T	**V**	GBLP 192–200	a
	F	**A**	Y	D	G	K	D	Y	**I**	MHC I α chain 140–148	a
	F	**P**	S	E	I	V	G	K	**R** **I**	Ribosomal protein S7/S8A 135–144	a
	M	**P**	W	F	K	G	w	K	**V**	Elongation factor 1a 208–216	a

References:
a: Falk et al. 1995a

Table 3 (Continued)
P HLA-B*5103

	Position									Comments	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		**A** **P** **G**	Y						V I F	Anchor at 9 deduced from individual ligands	a
Other preferred residues	T V D	F W	F D L	E L N R G Q T V	G A V N Q M R	I K T	V M				
										Source	
Examples for ligands	T	G	Y	L	N	T	V	T	**V**	GBLP 192–199	a
	D	A	H	I	Y	L	N	H	**I**	Thymidilate synthase 253–261	a
	Y	F	D	d	t	L	E	D	**F**	Unknown	a

References:
a: Falk et al. 1995a

Q HLA-B*5201

	Position									Comments	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		Q	F Y W		L I V			I V	I V	C-terminal anchor at 8 or 9	a
Other preferred residues	V L I	M F P	I L P D K	L I V P K E A	M F A T G	K N L T S	K E Q Y	M F	M F		
										Source	
Examples for ligands	T	G	Y	L	N	T	V	T	**V**	GBLP 192–200	a
	G	Q	F	K	T	Y	A	I		Ribos. prot. S21 60–67	a
	H	S	T	I	M	P	R	L		P1-CDC21 259–266	a
	G	F	Y	P	G	S	I	E	**V**	MHC II β chain 150–158	a
	V	Q	I	F	G	N	K	M		RBAP-2 266–273	a
	Y	P	D	P	A	N	G	K	**F**	Elongation factor 2 265–273	a
	L	Q	F	P	V	G	R	I		Histone 2a Z 25–32	a
	H	M	Y	I	F	L	H	T	**V**	Unknown	a

References:
a: Falk et al. 1995a

Table 3 (Continued)
R HLA-B53

	Position									Source	Ref.
	1	**2**	3	4	5	6	7	8	9		
Anchor residues		**P**									a
T-cell epitope	K	**P**	I	V	Q	Y	D	N	F	P. falciparum LSA-1 1786–1794	a

References:
a: Hill et al. 1992

S HLA-B*5801

	Position										Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**			
Anchor or auxiliary anchor residues		**A** **S** **T**		P E K	V I L M F				**F** **W**			a
Other preferred residues	K R I	G	G T I L V F Y N Q	D Q R	A D N T Y W Q	I V L F	L Y M N	N R K T	Y			
Examples for ligands	K	**A**	G	Q	V	V	T	I	**W**		Lamin C 490–498	a
	A	**G**	D	R	T	F	Q	K	**W**		MHC class I 260–268	a
	I	**t**	T	K	A	I	S	R	**F**		Unknown	a
	R	**T**	D	G	K	V	F	Q	**F**		Ribosomal protein L30 23–31	a
	I	**T**	S	Q	D	V	L	H	S	W	Cytochrome C oxidase 154–163	a
	I	**S**	D	S	N	P	F	L	T	Q	Unknown	a
	K	**t**	D	e	V	V	T	L	**F**		Unknown	a
	V	**T**	S	P	L	T	V	E	**W**		MHC class IIβ 209–217	a
	g	**A**	V	N	V	V	M	T	**f**		Glucose transporter 5 322–330	a

References:
a: Falk et al. 1995c

Table 3 (Continued)
T HLA-B60 (B*40012)

	Position									Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**		
Anchor or auxiliary anchor residues		**E**					I V		**L**		a
Other preferred residues			A V I L M F S D N	P K D G N Q T	L I V D T N P G K Q	K N P V I D R Q	L Y M	K R Q			
Examples for ligands	K	**E**	S	T	L	H	L	V	**L**		Ubiquitin 63–71	a
	H	**E**	A	T	L	R	c	w	**A**	**L**	MHC class I 221–230	a
	Y	**E**	I	H	D	G	M	N	**L**		HSHMO2C05	a
	S	**E**	S	P	I	V	V	V	**L**		Signal peptidase 45–54	a
	I	**E**	V	D	P	D	T	K	E	m **L**	Ribosomal protein S17 95–105	a

References:
a: Falk et al. 1995c

U HLA-B61 (B*4006)

	Position									Comments	Ref.
	1	**2**	3	4	5	6	7	8	**9**		
Anchor or auxiliary anchor residues		**E**	F I L V Y W			I			**V**		a
Other preferred residues	G R	P	M T	E G P S N D K A R N Q	V I L M D G V F N S K	N	Y V L W I T R D Q G	K S	A P	P1 deduced from individual ligands	
										Source	
Examples for ligands	G	**E**	F	G	G	F	G	S	**V**	IEF (mRNA) 9306 127–135	a
	E	**E**	F	Q	F	I	K	K	**A**	Associated-microfibril. protein 72–80	a
	G	**E**	F	V	D	L	Y	**V**		Ribosomal protein S21 6–13	a
	R	**E**	R	R	D	N	Y	**V**		Ribosomal protein S17 77–84	a
	R	**E**	I	I	I	N	A	**V**		Ribonucl. reductase 290–297	a
	G	**E**	F	S	I	T	Y	K		Ribosomal protein S15 116–123	a
	G	**E**	H	G	L	i	I	R	**V**	Unknown	a
	R	**E**	M	I	P	F	A	D	i	Unknown	a

References:
a: Falk et al. 1995c

Table 3 (Continued)
V HLA-B62 (B*1501)

	Position										Source	Ref.
	1	**2**	3	4	5	6	7	8	**9**			
Anchor or auxiliary anchor residues		**Q** **L**			I V				**F** **Y**			a
Other preferred residues	I	M V	K A N F P Y H R	P E G D	G L F T	V T G I	V T L I	Y V T				
Examples for ligands	V	**L**	K	P	G	M	V	V	T	**F**	Elongation factor 1 α 271–280	a
	Y	**L**	G	E	F	S	I	T	**Y**		Ribosomal protein S15 114–122	a
	G	**Q**	R	K	G	A	G	S	V		Ribosomal protein L8 (rat) 7–15	a
	K	**I**	K	S	F	V	K	V	**Y**		Ribosomal protein L27 66–74	a
	I	**Q**	P	G	R	G	F	V	L	**Y**	Unknown	a
	S	**Q**	F	G	G	G	S	Q	**Y**		Unknown	a
	G	**Q**	R	K	P	A	T	S	**Y**		Ribosomal protein L28 (rat) 68–76	a
	V	**Q**	G	P	V	G	L				Collagen α1 1106–1112	a
T-cell epitopes	I	**L**	G	N	K	I	V	R	M	**Y**	HIV gag 267–276	b

References:
a: Falk et al. 1995c; b: Buseyne et al. 1993

W HLA-B*7801

	Position								Comments	Ref.
	1	2	3	4	5	6	7	8		
Anchor or auxiliary anchor residues		**P** **A** **G**				I L F V		A	This motif is only partial; the C-terminal anchor has not been determined	a
Other preferred residues			Y D W	F D G L V S Q R N	D G V N R Q S T		A V N K Q E	K S		

References:
a: Falk et al. 1995a

Table 4 HLA-C motifs

A HLA-Cw*0301

	Position												Source	Ref.
	1	2	3	4	5	6	7	8	9					
Anchor or auxiliary anchor residues			V I Y L M	P		F Y			**L** **F** **M** **I**					a
Other preferred residues		A R	E N	E R	N	M	Q K S M	T						
T-cell epitopes	H	Q	A	I	S	P	R	T	**L**				HIV gag 144–152	b
	or Q	M	V	H	Q	A	I	S	P	R	T	**L**	HIV gag 141–152	

References:
a: Falk et al. 1993a; b: Littaua et al. 1991

B HLA-Cw*0401

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		**Y** **P** **F**				V I L			**L** **F** M		a
Other preferred residues			D H	D E P	A H M T R		A	K S H			
T-cell epitope	S	**F**	N	C	G	G	E	F	**F**	HIV-1 gp 120 380–388	b

References:
a: Falk et al. 1993a; b: Johnson et al. 1993

C HLA-Cw*0602

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues					I L F M	V I L			**L** **I** **V** **Y**		a
Other preferred residues	I F K Y	P R	P I G F Y K N A	P E D Q L	K	A T S	R K Q N	Y E Q N R G T S K			
Examples for ligands	Y	Q	F	T	G	I	K	K	**Y**	Unknown	a
	V	R	H	D	G	G	N	V	**L**	Unknown	a
	F	A	F	p	l	I	q	R	**V**	Unknown	a
	X	Q	r	T	P	k	A	g	l	Y **Y** Unknown	a

References:
a: Falk et al. 1993a

Table 4 (Continued)
D HLA-Cw*0702

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor or auxiliary anchor residues		Y P			V Y I L F M	V I L M			Y F L		a
Other preferred residues		R D	P G A	D E V Q P S G	T	A R	Y M N R V F E	E A F D K			
Examples for ligands	K	Y	F	D	E	H	Y	E	Y	CKS-2 11–19	a
	R	Y	R	P	G	T	V	A	L	Histone H3.3 40–48	a
	N	K	A	D	V	I	L	K	Y	Protein synthesis factor eIF-4C 87–95	a
	I	Y	P	q	n	v	i	L	Y	Unknown	a
	I	R	K	P	Y	I	w	E	Y	Glutamyl-tRNA synthetase 343–351	a
	N	Y	G	G	G	N	Y	G	S	G S Y Homologous hnRNP A2 or B1 (S11 = N) 277–288	a
	F	Y	P	P	y	l	Y			Unknown	a
	X	M	P	P	f	L	d	G		Unknown	a

References:
a: Falk et al. 1993a

Table 5 Processing motif for all MHC class II ligands

	Absolute position																	Ref.
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
		P										p	p	p	p	p		a, b, c

References:
a: Falk et al. 1994b; b: Kropshofer et al. 1993; c: Malcherek et al. 1993

Table 6 Human MHC class II motifs
A HLA-DRB1*0101

		Relative position									Source		Ref.
		1	2	3	**4**	5	**6**	7	8	**9**			
Anchor residues		**Y,V, L,F, I,A M,W**			**L,A I,V M,N Q**		**A,G S,T P**			L,A I,V N,F Y			a, b, e
Examples for ligands	VGSD	**W**	R	F	**L**	R	**G**	Y	H	Q	YA	HLA-A2 103–117	c
	VGSD	**W**	R	F	**L**	R	**G**	Y	H	Q	YAYDG	HLA-A2 103–120	c
	VGSD	**W**	R	F	**L**	R	**G**	Y	H	Q	Y	HLA-A2 103–116	c
	GSD	**W**	R	F	**L**	R	**G**	Y	H	Q	YA	HLA-A2 104–117	c
	LPKPPKPVSK	**M**	R	M	**A**	T	**P**	L	L	M	QALPM	Invariant chain 97–120	c
	IPAD	**L**	R	I	**I**	S	**A**	N	G	C	K	Na^+-K^+-ATPase 199–216	c
	RVE	**Y**	H	F	**L**	S	**P**	Y	V	S	PKESP	Transferrin receptor 680–696	c
	YKHT	**L**	N	Q	**I**	D	**S**	V	K	V	WPRRPT	Cattle fetuin 56–74	c
	AILE	**F**	R	A	**M**	A	Q	F	S	R	KTD	Unknown	d
	PK	**Y**	V	K	**Q**	N	**T**	L	K	L	AT*	Influenza HA 306–318	e

* Alignment determined by structural analysis
References:
a: Hammer et al. 1992; b: Falk et al. 1994b; c: Chicz et al. 1992; d: Kropshofer et al. 1992; e: Stern et al. 1994

Table 6 (Continued)
B HLA-DRB1*0301 (DR17)

		Relative position									Source		Ref.
		1	2	3	**4**	5	**6**	7	8	**9**			
Anchor or auxiliary anchor residues		**L,I F,M V**			**D**		K,R E,Q N			Y,L F			a, b, c
Examples for ligands	ISNQ	**L**	T	L	**D**	S	**N**	T	K	**Y**	FHKLN	Apolipoprotein B 2877–2894	a
	ISNQ	**L**	T	L	**D**	S	**N**	T	K	**Y**	FHKL	Apolipoprotein B 2877–2893	a
	ISNQ	**L**	T	L	**D**	S	**N**	T	K	**Y**	FHK	Apolipoprotein B 2877–2892	a
	VDT	**F**	L	E	**D**	V	**K**	N	L	**Y**	HSEA	α1-Antitrypsin 149–164	a
	KPRA	**I**	V	V	**D**	P	V	H	G	**F**	MY	LDL-Receptor 518–532	a
	KQT	**I**	S	P	**D**	Y	**R**	N	M	I		IgG2a, Membrane domain	a
	YPD	**F**	I	M	**D**	P	**K**	E	K	D	KV	Unknown	a
	NIQ	**L**	I	N	**D**	Q	**E**	V	A	R	FD	Unknown	a
	LLS	**F**	V	R	**D**	L	**N**	Q	Y	R	ADI	Transferrin receptor 618–632	a
	LPKPPKPVSK	**M**	R	M	A	T	P	L				Invariant chain 97–113	d, e, f
	LPKPPKPVSK	**M**	R	M	A	T	P	L	L	M	QALP	Invariant chain 97–119	d, e, f
	LPKPPKPVSK	**M**	R	M	A	T	P	L	L	M	QALPM	Invariant chain 97–120	d, e, f
	PKPPKPVSK	**M**	R	M	A	T	P	L				Invariant chain 98–113	d, e, f
	PKPPKPVSK	**M**	R	M	A	T	P	L	L	M	QA	Invariant chain 98–117	d, e, f
	KPPKPVSK	**M**	R	M	A	T	P	L	L	M	Q	Invariant chain 99–116	d, e, f
	KPPKPVSK	**M**	R	M	A	T	P	L	L	M	QALPM	Invariant chain 99–119	d, e, f
	VDDTQF	**V**	R	F	**D**	S	D	A	A	S	Q	HLA-A30 28-?	e
	ATKYGN	**M**	T	E	**D**	H	V	M	H	**L**	LQNA	Invariant chain 131–149	e
	VFLL	**L**	L	A	**D**	K	V	P	E	T	SLS	ACh receptor 289–304	e
	LNK	**I**	L	L	**D**	E	**Q**	A	Q	W	K	ICAM-2 64–76	e
	GPPKLD	**I**	R	K	**E**	E	**K**	Q	I	M	IDIFH	IFN-γ receptor 128–147	e
	GPPKLD	**I**	R	K	**E**	E	**K**	Q	I	M	IDIFHP	IFN-γ receptor 128–148	e
	GKFA	**I**	R	P	**D**	K	**K**	S	N	P	IIRTV	Cyt-b5 155–172	e
	YAN	**I**	L	L	**D**	R	**R**	V	P	Q	TDMTF	Apolipoprotein B 1207–1224	e
	NLF	**L**	K	S	**D**	G	**R**	I	K	**Y**	TLNKNSLK	Apolipoprotein B 1276–1295	e
	IPDNLF	**L**	K	S	**D**	G	**R**	I	K	**Y**	TLNKN	Apolipoprotein B 1273–1292	e
	IPDNLF	**L**	K	S	**D**	G	**R**	I	K	**Y**	TLNK	Apolipoprotein B 1273–1291	e
	IPDNLF	**L**	K	S	**D**	G	**R**	I	K	**Y**	TLN	Apolipoprotein B 1273–1290	a, e
	IPDNLF	**L**	K	S	**D**	G	**R**	I	K	**Y**	TL	Apolipoprotein B 1273–1289	e
	NLF	**L**	K	S	**D**	G	**R**	I	K	**Y**	TLNK	Apolipoprotein B 1276–1291	e
	NLF	**L**	K	S	**D**	G	**R**	I	K	**Y**	TLN	Apolipoprotein B 1276–1290	e
	VTT	**L**	N	S	**D**	L	**K**	Y	N	A	LDLTN	Apolipoprotein B 1294–1810	e
		V	G	S	**D**	W	**R**	F	L	R	GYHQYA	HLA-A2 103–117	e

References:
a: Malcherek et al. 1993; b: Geluk et al. 1994; c: Geluk et al. 1992; d: Riberdy et al. 1992; e: Chicz et al. 1993; f: Sette et al. 1992

Table 6 (Continued)
C HLA-DRB1*0401 (DR4Dw4)

		Relative position									Source		Ref.
		1	2	3	4	5	6	7	8	9			
Anchor or preferred residues		**F,Y** **W,I** L,V M			F,W I,L V,A D,E **no** **R,K**		N,S T,Q H,R	pol.* chg.* ali.*		pol.* ali.* K			a, b, c, d
Examples for ligands		**F**	V	R	**F**	D	**S**	**D**	A	**A**	SQRMEP	HLA-A2 33–47	a
	VDDTQ	**F**	V	R	**F**	D	**S**	**D**	A	**A**	SQRM	HLA-A2 28–45	a
		F	V	R	**F**	D	**S**	**D**	A	**A**	SQRM	HLA-A2 33–45	a
	VDDTQ	**F**	V	R	**F**	D	**S**	**D**	A	**A**	SPRGEP...	HLA-C 28–?	a
	DGKD	**Y**	I	A	**L**	N	E	**D**	L	**S**	S	HLA-B44 143–156	a
	LSS	**W**	T	A	**A**	D	**T**	**A**	A	**Q**	ITQ	HLA-B44 154–168	a
	LSS	**W**	T	A	**A**	D	**T**	**A**	A	**Q**	IT	HLA-B44 154–167	a
	IY	**F**	R	N	Q	K	G	**S**	H	**S**	GLQPTGFL	HLA-DR4β 252–270	a
	DVA	**F**	V	K	**D**	Q	**T**	**V**	I	**Q**	NTD	Cattle transferin 68–82	a
	YDHN	**F**	V	K	**A**	I	**N**	**A**	I	**Q**	KSW	Cathepsin C 170–185	a
	KHKV	**Y**	A	C	**E**	V	**T**	**H**	Q	**G**	...	Igκ chain C region 80–?	a
	HKV	**Y**	A	C	**E**	V	**T**	**H**	Q	**G**	L...	Igκ chain C region 81–?	a
	DGP	**F**	R	I	**I**	T	V	**P**	A	**A**	LDY	Unknown	a
	TGN	**Y**	R	I	**E**	S	V	**L**	S	**S**		Sphingolipid activator protein 3 165–176	a
	GERA	**M**	T	K	**D**	N	**N**	**L**	L	**G**	...	HSC 70 445–?	a
	XXX	**Y**	E	X	**A**	L	**S**	**L**	P	**S**	K...	Unknown	a
	GSLF	**V**	Y	N	**I**	T	**T**	**N**	K	Y	KAFLKQ	VLA-4 229–247	e
	SPEDF	**V**	Y	Q	**F**	K	G	**M**	C	Y	F	HLA-DQβ 3.2 chain 24–38	e
	AAPYEKEVP	**L**	S	A	**L**	T	**N**	**I**	L	**S**	AQL	PAI-1 261–281	e
	GVYF	**Y**	L	Q	**W**	G	**R**	**S**	T	**L**	VSVS	Ig heavy chain 121–?	e
	AEALERM	**F**	L	S	**F**	P	**T**	**T**	K	**T**		Cattle hemoglobin 26–41	e
	LRS	**W**	T	A	**A**	D	**T**	**A**	A	**Q**	ITQRKWEAA	HLA-Cw9 130–150	e
	DLSS	**W**	T	A	**A**	D	**T**	**A**	A	**Q**	ITQRKWEAA	HLA-Bw62 129–150	e
	APSP	**L**	P	E	T	T	E	**N**	V	**V**	CALG	HLA-DRα chain 182–198	e

* pol.: Polar; chg.: charged; ali.: aliphatic
References:
a: Friede and co-workers, submitted; b: Sette et al. 1993; c: Hammer et al. 1993; d: Hill et al. 1994; e: Chicz et al. 1993

D HLA-DRB1*0402 (DR4Dw10)

		Relative position									Source		Ref.
		1	2	3	4	5	6	7	8	9			
Anchor or preferred residues		**V,I** **L,M**			Y,F W,I L,M R,N **no** **D,E**		**N,Q** **S,T** **K**	R,K H,N Q,P; rare D,E		pol.* ali.* **H**			a
Examples for ligands	GPDGR	**L**	L	R	G	H	**N**	**Q**	F	**A**	YDGKD	HLA-B38 128–146	a
	GR	**L**	L	R	G	H	**N**	**Q**	F	**A**	YDGK	HLA-B38 131–145	a
	I	**I**	K	G	V	R	**K**	**S**	N	**A**	AERRG	HLA-DRα 238–252	a
		I	Y	F	**R**	N	**Q**	**K**	G	**H**	SGLQPTGFLS	DR4β 248–266	a
				F	**R**	N	**Q**	**K**	G	**H**	SGLQP	DR4β 250–261	a
	F	**I**	Y	F	**R**	N	**Q**	**K**	G	**H**	SGLQPTGFLS	DR4β 249–266	a
		Y	V	R	**F**	D	**S**	D	V	**G**	EY	DR4Dw10β 37–47	a
	LPKPPKPVSK	**M**	R	M	A	T	P	L	L	**Q**		Invariant chain 97–?	a
	FDQK	**I**	V	E	**W**	D	**S**	**R**	K	**S**	KYFE	BLAST-1 62–78	a
	DQK	**I**	V	E	**W**	D	**S**	**R**	K	S	KYF	BLAST-1 63–77	a
	IKI	**I**	S	K	**I**	E	**N**	**H**	E	**G**	VRR	Pyruvate kinase 264–278	a
	IKI	**I**	S	K	**I**	E	**N**	**H**	E	**G**	VR	Pyruvate-kinase 264–277	a
	FGR	**I**	G	R	**L**	V	**T**	**R**	A	**A**	FNSG	GAPDH 11–25	a
	FGR	**I**	G	R	**L**	V	**T**	**R**	A	**A**	FN	GAPDH 11–23	a
	GFGR	**I**	G	R	**L**	V	**T**	**R**	A	**A**	FNSG	GAPDH 10–25	a
	CNE	**I**	I	N	**W**	L	D	**K**	N	**Q**		HSC 70 574–585	a
	QPD	**L**	R	Y	**L**	F	L	N	G	**N**		Leucine-rich α2-glycoprotein 200–211	a

References:
a: Friede and co-workers, submitted

Table 6 (Continued)
E HLA-DRB1*0404 (DR4Dw14)

		Relative position									Source	Ref.
		1	2	3	4	5	6	7	8	9		
Anchor or preferred residues		**V,I L,M**			F,Y W,I L,V M,A D,E **no R,K**		N,T S,Q R	pol.* chg.* ali.*		pol.* ali.* K		a
Examples for ligands	GSHS	**M**	R	Y	**F**	H	**T**	**A**	M	**S**	RPGRGE	HLA-B60 1–?	a
	SHS	**M**	R	Y	**F**	H	**T**	**A**	M	**S**	RPGRGE	HLA-B60 2–?	a
	YDNS	**L**	K	I	**I**	S	**N**	**A**	S	C	TTN	GAPDH 139–154	a

* pol.: Polar; chg.: charged; ali.: aliphatic
References:
a: Friede and co-workers, submitted

F HLA-DRB1*0405 (DR4Dw15)

		Relative position									Source	Ref.
		1	2	3	4	5	6	7	8	9		
Anchor or preferred residues		**F,Y W,V I,L M**			V,I L,M D,E		N,S T,Q K,D	pol.* chg.* ali.*		**D,E** Q		a
Examples for ligands	YPTQRAR	**Y**	Q	W	**V**	R	C	**N**	P	**D**	SNS	PGSG 1–19	a
	QRAR	**Y**	Q	W	**V**	R	C	**N**	P	**D**	SNS	PGSG 4–19	a
	RAR	**Y**	Q	W	**V**	R	C	**N**	P	**D**	SNS	PGSG 5–19	a
	KPPQ	**Y**	I	A	**V**	H	V	**V**	P	**D**	Q	MIF 32–45	a
	FRE	**F**	K	L	**S**	K	V	**W**	R	**D**	QH	Transferrin receptor 173–186	a
	FRE	**F**	K	L	**S**	K	V	**W**	R	**D**	Q	Transferrin receptor 173–185	a
	RE	**F**	K	L	**S**	K	V	**W**	R	**D**	QH	Transferrin receptor 174–186	a
	RE	**F**	K	L	**S**	K	V	**W**	R	**D**	Q	Transferrin receptor 174–185	a
	VEPDH	**Y**	V	V	**V**	G	A	**Q**	R	**D**	A	Transferrin receptor 397–411	a
	EPDH	**Y**	V	V	**V**	G	A	**Q**	R	**D**	A	Transferrin receptor 398–411	a
	THY	**Y**	A	V	**A**	V	V	**K**	K	**D**	TDFK	Transferrin 92–107	a
	KELK	**I**	D	I	**I**	P	**N**	**P**	Q	**E**	R	Hsp 90-beta 68–81	a
	YLL	**Y**	Y	T	**E**	F	**T**	**P**	T	**E**	KD	β_2-microglobulin 83–96	a
	LL	**Y**	Y	T	**E**	F	**T**	**P**	T	**E**	KDEY	β_2-microglobulin 84–98	a
	CAIHAKR	**V**	T	I	**M**	P	**K**	**D**	I	**Q**	LA...	Histone H3 110–?	a
	APNT	**F**	K	T	**L**	D	**S**	W	R	**D**		ras-related protein RAB-7 (rat) 86–98	a
	VADK	**I**	Q	L	**I**	N	**N**	**M**	L	**D**		Phosphoglycerate kinase 216–228	a
	GSTV	**F**	D	N	**L**	P	**N**	**P**	E	I	DGDYYGW	Unknown	b
	XXXQ	**Y**	I	A	**V**	H	V	**V**	P	**D**	QT	Homol. MIF 32–46	b
	SDPIL	**Y**	R	P	**V**	A	V	**A**	L	**D**		PKM2 99–112	b
		V	P	I	Q	R	A	**V**	Y	**Q**	NVVVNNPXD	Unknown	b
	SPGTGA	**Y**	Y	V	**L**	L	N	...				Unknown	b
	KPPQ	**Y**	I	A	**V**	H	V	**V**	P	**D**	QLM	MIF 32–47	c
	KPPQ	**Y**	I	A	**V**	H	V	**V**	P	**D**	QL	MIF 32–46	c
	KPPQ	**Y**	I	A	**V**	H	V	**V**	P	**D**	Q	MIF 32–45	c
	DPIL	**Y**	R	P	**V**	A	V	**A**	L	**D**	TKGPE	PKM2 101–118	c
	DPIL	**Y**	R	P	**V**	A	V	**A**	L	**D**	TKGP	PKM2 101–117	c
	DNPQTHY	**Y**	A	V	A	V	V	**K**	K	**D**	TDFKL	Transferrin 88–108	c
	DNPQTHY	**Y**	A	V	A	V	V	**K**	K	**D**	TDFK	Transferrin 88–107	c
	NPQTHY	**Y**	A	V	A	V	V	**K**	K	**D**	TDFKL	Transferrin 89–108	c
	NPQTHY	**Y**	A	V	A	V	V	**K**	K	**D**	TDFK	Transferrin 89–107	c
	DNPQTHY	**Y**	A	V	A	V	V	**K**	K	**D**		Transferrin 88–103	c
	THY	**Y**	A	V	A	V	V	**K**	K	**D**	TDF	Transferrin 92–106	c
	LL	**Y**	Y	T	**E**	F	**T**	**P**	T	**E**	KDEY	β_2m 84–98	c
	L	**Y**	Y	T	**E**	F	**T**	**P**	T	**E**	KD	β_2m 85–26	c
	XXXXKK	**V**	V	V	Y	L	**Q**	K	L	**D**	T	Cathepsin C 58–73	c
	KK	**V**	V	V	Y	L	**Q**	K	L	**D**	TAYD	Cathepsin C 62–76	c
	K	**V**	V	V	Y	L	**Q**	K	L	**D**	TAYD	Cathepsin C 63–76	c
	KP	**Y**	N	E	A	K	**T**	X	F	**D**	KY	Apolipoprotein B-100 3218–3230	c

* pol.: Polar; chg.: charged; ali.: aliphatic
References:
a: Friede and co-workers, submitted; b: Matsushita et al. 1994; c: Kinouchi et al. 1994

Table 6 (Continued)
G HLA-DRB1*1101

		Relative position										Source	Ref.
		1	2	3	**4**	5	**6**	7	8	9			
Anchor residues		W,Y F			M,L V,I		R,K						a, b
Examples for ligands	IDF	**Y**	T	S	**I**	T	**R**	A	R	F	EE	HSC 70 291–305	b
	CPAG	**Y**	T	C	N	V	**K**	A	R	S	CEK	Granulin D 41–56	b
	VNH	**F**	I	A	E	F	**K**	R	K	H	KKD	Homol. HSC 70 238–252	b
	VNH	**F**	I	A	E	F	**K**	R	K	H	K	Homol. HSC 70 238–250	b
	MR	**Y**	F	H	T	S	V	S	R	P	GRGEP	HLA-Bw61 5–20	b
	KHKV	**Y**	A	C	E	V	T	H	Q	G	LS	Homol. Ig κ-chain 190–204	b

References:
a: Hammer et al. 1993; b: Newcomb and Cresswell 1993

H HLA-DRB1*1201

		Relative position										Source	Ref.
		1	2	**3**	4	5	**6**	7	8	**9**			
Anchor residues		**I,L** F,Y V		**L,M N,V A**			V,Y F,I N,A			**Y,F M,I V**			a
Examples for ligands	GPDGRL	**L**	R	G	Y	D	Q	F	A	**Y**	DGK	HLA-B38 104–121	a
	GPDGRL	**L**	R	G	H	N	Q	Y	A	**Y**	D	HLA class I 104–119	a
	TGT	**I**	K	**L**	L	N	E	N	S	**Y**	VP	Transferrin receptor 142–155	a
	T	**I**	K	**L**	L	N	E	N	S	**Y**	VPR	Transferrin receptor 144–156	a
	FTGT	**I**	K	**L**	L	N	E	N	S	**Y**	VPR	Transferrin receptor 141–156	a
	DFTGT	**I**	K	**L**	L	N	E	N	S	**Y**	VPR	Transferrin receptor 140–156	a
	SDEK	**I**	R	**M**	N	R	**V**	V	R	N	NLR	Valosin-cont. protein p97 78–93	a
	SSV	**I**	T	**L**	N	T	**N**	V	G	**L**	YXQT	Homol. to apolipoprotein	a
	EAL	**I**	H	Q	L	K	**I**	N	P	**Y**	VLS	Unknown	a
	AHL	**F**	K	Q	N	K	**V**	V	H	**V**	NG	Dihydrolipoamide dehydrogenase 138–152	b

References:
a: Falk et al. 1994b; b: Falk and co-workers, unpublished

I HLA-DRB1*1501 (DR2b)

		Relative position										Source	Ref.
		1	2	3	**4**	5	6	**7**	8	9			
Anchor residues		L,V I			F,Y I			I,L V,M, F					a, b
Examples for ligands	EAEQ	**L**	R	A	**Y**	L	D	G	T	G	VE	HLA-A3 152–166	a
		L	E	E	**F**	G	R	**F**	A	S	FEAQG	HLA-DRα 45–58	a
	D	**V**	G	V	**Y**	R	A	**V**	T	P	QGRPDA	HLA-DQw6 43–58	a
T-cell epitope	PV	**V**	H	F	**F**	K	N	**I**	V	T		MBP 85–95	b

References:
a: Vogt et al. 1994; b: Wucherpfennig et al. 1994

Table 6 (Continued)
K HLA-DRB5*0101 (DR2a)

		Relative position										Source	Ref.
		1	2	3	4	5	6	7	8	9			
Anchor or preferred residues		**F,Y** L,M			Q,V I,M					**R,K**			a, b
Examples for ligands	DVGV	**Y**	R	A	**V**	T	P	Q	G	**R**	P	HLA-DQw6 43–56	a
	DVGV	**Y**	R	A	**V**	T	P	Q	G	**R**	PDA	HLA-DQw6 43–58	a
	DSDVGV	**Y**	R	A	**V**	T	P	Q	G	**R**	PD	HLA-DQw6 41–57	a
	DSDVGV	**Y**	R	A	**V**	T	P	Q	G	**R**	PDA	HLA-DQw6 41–58	a
	DSDVGV	**Y**	R	A	**V**	T	P	Q	G	**R**	PDAEY	HLA-DQw6 41–60	a
	AAD	**M**	A	A	**Q**	I	T	K	R	**K**	WEAAH	HLA-A3 135–151	a
	TAAD	**M**	A	A	**Q**	I	T	K	R	**K**	WEA	HLA-A3 134–149	a
	DVGE	**F**	A	A	**V**	T	E	K	R	**R**	PDAEYW	HLA-DR2b 43–61	a
T-cell epitopes	PK	**Y**	V	K	**Q**	N	T	L	**K**	L	AT	HA 307–319	c
		L	Q	A	A	P	A	L	**D**	**K**	L	HSP65 418–427	a, d
	VHF	**F**	K	N	**I**	V	T	P	**R**	T	P	MBP 87–99	e
	ASD	**Y**	K	S	A	H	K	G	F	**K**	GVD	MBP 131–145	a
	KG	**F**	K	G	**V**	D	A	Q	G	T	LSKI	MBP 139–153	a

References:
a: Vogt et al. 1994; b: Wucherpfennig et al. 1994; c: O'Sullivan et al. 1991; d: Anderson et al. 1988; e: Martin et al. 1991

L HLA-DQA1*0501/DQB1*0301

		Relative position										Source	Ref.
		1	2	3	4	5	6	7	8	9			
Anchor residues		**F,Y I,M** L,V				**V,L** I,M Y		Y,F M,L V,I					a
Preferred residues	A		A	A	A								
Examples for ligands	TPL	**L**	M	Q	A	**L**	P	**M**	G	A	LPQG	Invariant chain 111–126	a
	TPL	**L**	M	Q	A	**L**	P	**M**	G	A	LPQ	Invariant chain 111–125	a
	KPPKPVSKMR	**M**	A	T	P	**L**	L	**M**	Q	A		Invariant chain 99–117	a
	LPKPPKPVSKMR	**M**	A	T	P	**L**	L	**M**				Invariant chain 97–115	a
	IPE	L	N	K	V	A	R	A	A	A		Transferrin receptor 579–597	a
	DVEV	**Y**	R	A	V	T	P	**L**	G	P	EVAGQF	DQβ chain 43–55	a

References:
a: Falk et al. 1994b

M HLA-DPA1*0201/DPB1*0401

		Relative position										Source	Ref.
		1	2	3	4	5	6	7	8	9	10		
Anchor residues		**F,L Y,M** I,V A						**F,L Y,M** V,I A			**V,Y I,A** L		a
Examples for ligands	EKK	**Y**	F	A	A	T	Q	**F**	E	P	**L**	AARL	Unknown	a
	KK	**Y**	F	A	A	T	Q	**F**	E	P	**L**	AARL	Unknown	a
	EKK	**Y**	F	A	A	T	Q	**F**	E	P	**L**		Unknown	a
	GPG	**A**	P	A	D	V	Q	**Y**	D	L	**Y**	LNVANRR	IL-3 Receptor α-chain 127–146	a

References:
a: Falk et al. 1994b

Table 6 (Continued)
N HLA-DPA1*0102/DPB1*0201

		Relative position									Source		Ref.
		1	2	3	4	5	6	7	8	9			
Anchor residues		**F,L M,V W,Y**				**F,L M,Y**			**I,A M,V**				a
Examples for ligands	ADEKKF	**W**	G	K	Y	**L**	Y	E	**I**	A	RRHP	Cattle serum albumin 152–170	a
	GEP	**L**	S	Y	T	R	F	S	**L**	A	RQVDG	Transferrin receptor 15–31	a
	LPSQA	**F**	E	Y	I	L	Y	N	K	G		Cathepsin H 185–198	a

References:
a: Rötzschke and Falk 1994

Table 7 Other human class II ligands

MHC molecule	Peptide sequence	Source		Ref.
HLA-DR2 (DRB5*0101 or DRB1*1501)	NIVIKRSNSTAATNEVPEVTVFS	HLA-DQα	97– 119	a
	NIVIKRSNSTAATNEV	HLA-DQα	97– 112	a
	SDVGVYRAVTPQGRPDAE	HLA-DQβ	42– 59	a
	DVGVYRAVTPQGRPDAE	HLA-DQβ	43– 59	
	DVGVYRAVTPQGRPD	HLA-DQβ	43– 57	
	RVQPKVTVYPSKTQPLQH	HLA-DRB1*1501	94– 111	a
	RVQPKVTVYPSKTQP	HLA-DRB1*1501	94– 108	a
	LSPIHIALNFSLDPQAPVDSHGLRPALHYQ	Fibronectin receptor α	586– 616	a
	DGILYYYQSGGRLRRPVN	K+ channel protein	173– 190	a
	IQNLIKEEAFLGITDEKTEG	Mannose binding protein	174– 193	a
	EHHIFLGATNYIYVLNEEDLQKV	MET protooncogene	59– 81	a
	QELKNKYYQVPRKGIQA	Guanylate binding protein 2	434– 450	a
	FPKSLHTYANILLDRRVPQTD	Apolipoprotein B100	1200–1220	a
	FPKSLHTYANILLDRRVPQ	Apolipoprotein B100	1200–1218	a
	LWDYGMSSSPHVLRNR	Factor VIII	1775–1790	a
HLA-DRB1*0701	RPAGDGTFQKWASVVVPSGQ	HLA-A29	234– 253	a
	RPAGDGTFQKWASVVV		234– 249	a
	GDGTFQKWASVVVPSGQEQRYT		237– 258	a
	GDGTFQKWASVVVPSGQE		237– 254	a
	GTFQKWASVVVPSG		239– 252	a
	GTFQKWASVVVPSGQ		239– 253	a
	GTFQKWASVVVPSGQEQRYTCHV		239– 261	a
	RETQISKTNTQTYRENL	HLA-B44	83– 99	a
	RETQISKTNTQTYREN		83– 98	a
	RETQISKTNTQTYRE		83– 97	a
	RSNYTPITNPPEVTVLTNSPVELREP	HLA-DR α chain	101– 126	a
	GALANIAVDKANLEIMTKRSN		58– 78	a
	SLQSPITVEWRAQSESAQSKMLSGIGGFVL	HLA-DQ α chain	179–?	a
	VTQYLNATGNRWCSWSLSQAR	4F2	318– 338	a
	VTQYLNATGNRWCSWSL		318– 334	a
	TSILCYRKREWIK	LIF receptor	854– 866	a
	PAFRFTREAAQDCEV	Thromboxane-A synthase	406– 420	a
	GDMYPKTWSGMLVGALCALAGVLTI	K+ channel protein	492– 516	a
	TPSYVAFTDTERLIGDA	Hsp 70	38– 54	a
	TPSYVAFTDTERLIG		38– 52	a
	VPGLYSPCRAFFNKEELL	EBV MCP	1264–1282	a
	VPGLYSPCRAFFNK		1264–1277	a
	KVDLTFSKQHALLCSDYQADYES	Apolipoprotein B 100	1586–1608	a
	KVDLTFSKQHALLCS		1586–1600	a
	FSHDYRGSTSHRL		1942–1954	a
	LPKYFEKKRNTII		2077–2089	a
	APVLISQKLSPIYNLVPVK	Complement C9	465– 483	a
	VGSDWRFLRGYHQYAYDG	HLA-A2	103– 120	a
	PKPPKPVSKMRMATPLLMQALP	Invariant chain	98– 119	a
	APSPLPETTENVVCALGLTV	HLA-DRα chain	182– 200	a
	KHKVYACEVTHQGL	Ig kappa chain	188– 201	a

Table 7 (Continued)

MHC molecule	Peptide sequence	Source		Ref.
HLA-DRB1*0801	APSPLPETTENVVCALG	HLA-DRα chain	182– 198	a
	SETVFLPREDHLFRKFHYLPFLP	HLA-DR α chain	158– 180	a
	RHNYELDEAVTLQ	HLA-DP β chain	80– 92	a
	DPQSGALYISKVQKEDNSTYI	LAM Blast-1	88– 108	a
	GALYISKVQKEDNSTYI		92– 108	a
	DPVPKPVIKIEKIEDMDD		129– 146	a
	DPVPKPVIKIEKIED		129– 143	a
	FTFTISRLEPEDFAVYYC	Ig κ chain	63– 80	a
	FTFTISRLEPEDFAV		63– 77	a
	DPVEMRRLNYQTPG	LAR	1302–1316	a
	YQLLRSMIGYIEELAPIV	LIF receptor	709– 726	a
	GNHLYKWKQIPDCENVK	IFN-α receptor	271– 287	a
	LPFFLFRQAYHPNNSSPVCY	IL-8 receptor	169– 188	a
	RPSMLQHLLR	Ca^{2+} release channel	2614–2623	a
	DDFMGQLLNGRVLFPVNLQLGA	CD35	359– 380	a
	IPRLQKIWKNYLSMNKY	CD75	106– 122	a
	EPFLYILGKSRVLEAQ	Calcitonin receptor	38– 53	a
	NRSEEFLIAGKLQDGLLH	TIMP-1	101– 118	a
	RSEEFLIAGKLQDGLL		102– 117	a
	SEEFLIAGKLQDGLL		103– 117	a
	NRSEEFLIAGKL		101– 112	a
	QAKFFACIKRSDGSCAWYRGAAPPKQEF	TIMP-2	187– 214	a
	QAKFFACIKRSDGSCAWYR		187– 205	a
	DRPFLFVVRHNPTGTVLFM	PAI-1	378– 396	a
	MPHFFRLFRSTVKQVD		133– 148	a
	QNFTVIFDTGSSNLWVPSVYCTSP	Cathepsin E	89– 112	a
	QNFTVIFDTGSSNLWV		89– 104	a
	TAFQYIIDNKGIDSDAS	Cathepsin S	189– 205	a
	DEYYRRLLRVLRAREQIV	Cystatin SN	41– 58	a
	EAIYDICRRNLDIERPT	Tubulin α-1 chain	207– 223	a
	EAIYDICRRNLDI		207– 219	a
	HELEKIKKQVEQEKCEIQAAL	Myosin β heavy chain	1027–1047	a
	AEVYHDVAASEFF ...	α-enolase	23–?	a
	KRSFFALRDQIPDL	c-*myc*	371– 385	a
	RQYRLKKISKEEKTPGC	K-ras	164– 180	a
	KNIFHFKVNQEGLKLSNDMM	Apolipoprotein B-100	1724–1743	a
	KNIFHFKVNQEGLKLS		1724–1739	a
	YKQTVSLDIQPYSLVTTLNS		1780–1799	a
	STPEFTILNTLHIPSFT		2646–2662	a
	TPEFTILNTLHIPSFTID		2647–2664	a
	TPEFTILNTLHIPSFT		2647–2662	a
	SNTKYFHKLNIPQLDF		2885–2900	a
	LPFFKFLPKYFEKKRNT		2072–2088	a
	LPFFKFLPKYFEKKR		2072–2086	a
	WNFYYSPQSSPDKKL		4022–4036	a
	DVIWELLNHAQEHFGKDKSKE	Cattle transferrin	261– 281	a
	DVIWELLNHAQEHFG		261– 275	a
	DVIWELLNHAQEH		261– 273	a
	IALLLMASQEPQRMSRNFVR	von Willebrand factor	617– 636	a
	IALLLMASQEPQRM		617– 630	a
HLA-DR11 or Dw52	SXVITLNTNVGLYXQS	Homol. Apolipoprotein	3345–3360	b
	DPXQDELQKLNAXDP	Unknown		b
	XPELNKVARAAAEVAG	Homol. Transferrin receptor	580– 595	b
DR17 or DRw 52	TFDEIASGFRQGGASQ	Glucose transporter	459– 474	a
	YGYTSYDTFSWAFL	Na+ channel protein	384– 397	a
	GQVKKNNHQEDKIE	CD45	1071–1084	a
	TGHGARTSTEPTTDY	EBV gp220	592– 606	a
	KELKRQYEKKLRQ	EBV tegument p140	1395–1407	a
	SPLQALDFFGNGPPVNYKTGNL	IP 30	38– 59	a

References:
a: Chicz et al. 1993; b: Newcomb and Cresswell 1993

Table 8 Mouse class II motifs

A H-2E^k

		Relative position									Source	Ref.
		1	2	3	4	5	6	7	8	9		
Anchor or preferred residues		I,L V,F Y,W			I,L V,F S		Q,N A			K,R		a, b, c
Examples for ligands	HPPHIE	I	Q	M	L	K	N	G	K	K		β_2m 42–56	c
	DNRM	V	H	F	I	A	E	F	K	R	K	HSC70 234–248	c
	TPTL	V	E	A	A	R	N	L	G	R	VG	Serum albumin 347–361	c
	VNKE	I	Q	N	A	V	Q	G	V	K	HI	C cyt inhib. 41–55	c
	GFPT	I	Y	F	S	P	A	N	K	K	L	ER60 448–461	a
	IP	L	I	M	L	I	N	K	A	R	NKAE	Unknown	a
	YDRN	T	K	S	P	L	F	V	G	K	V	α1-antitryp. 397–410	a
		F	A	E	F	G	T	L	K	K	AAVHYDRSG	Unknown	a
	LH	L	G	Y	L	P	N	Q	L	F	R	(human) dead box protein	a
	IPGGP	V	R	L	C	P	G	R	I	R		Cattle fetuin 342–	a
T-cell epitopes	RADL	I	A	Y	L	K	Q	A	T	K		MCC 91–103	b
	RADL	I	A	Y	L	K	Q	A	T	A	K	PCC 91–104	b
	LEDARR	L	K	A	I	Y	E	K	K	K		λrep 12–26	e
	QD	I	L	I	R	L	F	K	S	H	PETL	SWMb 26–40	e
	VTV	L	T	A	L	G	A	I	L	K	K	SWMb 66–78	d
		L	T	A	L	G	G	I	L	K		EqMb 69–77	b
		L	T	A	L	G	T	I	L	K		MoMb 69–77	b
		I	T	A	F	N	E	G	L	K		MoHb 68–76	b
	KVFGR	C	E	L	A	A	A	M	K	R	HGLD	HEL 1–18	e
	SALLSSD	I	T	A	S	V	N	C	A	K		HEL 81–96	d
		W	V	A	W	R	N	R	C	K	GTD	HEL 108–119	d
	VEK	Y	G	P	E	A	S	A	F	T	KKMVENAK	SNase 51–70	e
	RTDKYGRG	L	A	Y	I	Y	A	D	G	K	MVN	SNase 81–100	e
	HEHQ	L	R	K	S	E	A	Q	A	K	KEKLNIW	SNase 121–140	f
		I	A	K	F	G	T	A	F	K		LLO 218–226	b

References:
a: Schild and co-workers, submitted; b: Reay et al. 1994; c: Marrack et al. 1993; d: Spouge et al. 1987; e: Altuvia et al. 1994; F: Sette et al. 1989

B H-2E^d

		Relative position									Source	Ref.
		1	2	3	4	5	6	7	8	9		
Anchor or preferred residues		W,Y F,I, L,V			K,R I		I,L V,G			K,R		a
Examples for ligands	SQLELR	W	K	S	R	H	I	K	E	R		IL-2R. γ chain 168–182	a
	LELR	W	K	S	R	H	I	K	E	R		IL-2R. γ chain 170–182	a
	ERAEA	W	R	Q	K	L	H	G	R	L		Apo-E prec. 222–236	a
	RAEA	W	R	Q	K	L	H	G	R	L		Apo-E prec. 223–236	a
	AQ	F	M	W	I	I	R	K	R	I	QLP	Unknown	a
	SLDEH	Y	H	I	R	V	H	L	V	K		Similar Apolipoprotein B 2211–2224	a
	GQFY	F	L	I	R	K	R	I	H	L	R	C. elegans cDNA homol. 74–87	a
	LV	V	D	N	G	S	G	M	C	K	AGF	Actin B 8–21	a
T-cell epitopes	ALWFRNH	F	V	F	G	G	G	T	K	V	TV	Ig lambda 91–108	b
	KYLEFISEA	I	I	H	V	L	H	S	R			SWM 102–118	c
	NKALE	L	F	R	K	D	I	A	A	K	Y	SWM 132–146	d
	W	V	A	W	R	N	R	C	K	G	TD	HEL 108–119	c
	A	Y	V	Y	K	P	N	N	T	H	EQHLRKSE	SNase 112–129	e
	SS	F	E	R	F	E	I	F	P	K		FLU PR/8 HA 109–119	c
	LEDARR	L	K	A	I	Y	E	K	K	K		λrep 12–26	c
	EK	I	R	L	R	P	G	G	K	K	K	HIV-1 gag p17 17–28	f

References:
a: Schild and co-workers, submitted; b: Bogen et al. 1986; c: Spouge et al. 1987; d: O'Sullivan et al. 1991; e: Chicz et al. 1992; f: Sette et al. 1989

Table 8 (Continued)
C H-2E^s

		Relative position									Comments	Ref.
		1	2	3	4	5	6	7	8	9		
Anchor or preferred residues		**I,V L**			L,I V		Q,N			**K,R**	This motif has been predicted based on prediction of pocket structure and comparison with H-2E^k and H-2E^d motifs	a
											Source	
Examples for ligands		**L**	Y	V	**L**	K	**I**	G	K	**K**	DG	Carboxypeptidase A 44–54	b
	HPPHIE	**I**	Q	M	**L**	K	**N**	G	K	**K**		β_2 42–56	b
	EGEC	V	E	W	**L**	H	R	Y	L	**K**	NG	H-2L^d 160–174	b
	MQKEITA	**L**	A	P	**S**	T	M	K	I	**K**	II	β-actin 286–303	b
	CT	F	A	I	**C**	W	L	P	F	**H**	VFFL	Substance P receptor 255–269	b
	EGSLI	**V**	E	K	**I**	M	**Q**	S	S	S	E	HSP60 478–492	b
T-cell epitope	DL	**I**	A	Y	**L**	K	**Q**	A	T	**K**		MCC 93–103	c, d

References:
a: Schild and wo-workers, submitted; b: Marrack et al. 1993; c: Altuvia et al. 1994; d: Reay et al. 1994

D H-2E^b

		Relative position									Comments	Ref.
		1	2	3	4	5	6	7	8	9		
Anchor or preferred residues		**W,F Y**			L,I F,V		Q,N, A			**K,R**	This motif has been predicted based on prediction of pocket structure and comparison with H-2E^k and H-2E^b motifs	a
											Source	
Examples for ligands	SPSYV	**Y**	H	Q	**F**	E	R	R	A	**K**	YK	MuLV env protein 454–469	b
	SPSYV	**Y**	H	Q	**F**	E	R	R	A	**K**	YKREPVSL	MuLV env protein 454–475	b
	SPSYV	**Y**	H	Q	**F**	E	R	R	A	**K**		MuLV env protein 454–467	b
	GK	**Y**	L	Y	E	I	**A**	R	R	**H**	PYFY	BSA 141–155	b
	XPQS	**Y**	L	I	H	E	X	X	X	I	S	Unknown	b
T-cell epitopes	RTDKYGRG	**L**	A	Y	**I**	Y	**A**	D	G	**K**	MVN	SNase 81–100	c, d
	DL	**I**	A	Y	**L**	K	**Q**	A	T	**K**		MCC 93–103	c, d

References:
a: Schild and co-workers, submitted; b: Rudensky et al. 1991; c: Altuvia et al. 1994; d: Reay et al. 1994

Table 9 Other mouse class II ligands

MHC Molecule	Peptide sequence	Source		Ref.
H-2A^b	HNEGFYVCPGPHRP	MuLV env	145–158	a
	ASFEAQGALANIAVDKA	H-2Eα	52– 68	a
	KPVSQMRMATPLLMR	Invariant chain	86–100	a
	NYNAYNATPATLAVD	Unknown		a, b
	RPDAEYWNSQPE	H-2Aβ	55– 66	b
	XNADFKTPATLTVDKP	IgG V_μ	59– 74	b
H-2A^s	IRLKITDSGPRVPIGPN	MuLV env	255–269	b
	IRLKITDSGPRVPIG	MuLV env	255–267	b
	WQSQSITCNVAHPASST	IgG2a	194–210	b
	NVEVHTAQTQTHREDY	IgG2a	281–296	b
	KPTEVSGKLVHANFGT	Transferrin receptor	203–218	b
	XPYMFADKVVHLPGSQ	Unknown		b
H-2A^d	WANLMEKIQASVATNPI	Apo-E	268–284	c
	WANLMEKIQASVATNP	Apo-E	268–283	c
	DAYHSRAIQVVRARKQ	Cys-C	40– 55	c
	ASFEAQGALANIAVDKA	H-2I-Eα^d	52– 68	c
	ASFEAQGALANIAVDK	H-2I-Eα ^{o}^{d}	52– 67	c
	EEQTQQIRLQAEIFQAR	Apo-E	236–252	c
	EQTQQIRLQAEIFQAR	Apo-E	237–252	c
	KPVSQMRMATPLLMRPM	Li	85–101	c
	VPQLNQMVRTAAEVAGQX	Tf recp.	442–459	c
	ISQAVHAAHAEINE	Ovalbumin	323–336	c
	LEDARRLKAIYEKKK	λ repressor	12– 26	c
H-2A^k	DGSTDYGILQINSR	Hen egg lysozyme	48– 61	d
	DGSTDYGILQINS		48– 60	d
	DGSTDYGILQINSRW		48– 62	d
	DYGILQINSRWW (C)		52– 63 (64)	d
	IIANDQGNRTTPSY	hsp70	28– 41	d
	TPRRGEVYTCHVEHP	H-2I-A^k β chain	165–179	d
	KVHGSLARAGKVRGQTPKVAKQ	S30 ribosomal protein	75– 96	d
	AGKVRGQTPKVAKQEKKKKKT		83–103	d
	EPLVPLDNHIPENAQPG	Ryudocan	84–100	d
	XQLGAQNEMLXPL	Unknown		e
	XXKKGTDFQLNQLE	Transferrin	100–113	e
	KGTDFQLNQLEGKKG	Transferrin	103–117	e
	YVRFDSFVGEYRAVT	H-2Aβ^k	37– 51	e
	XPLALQFAELPVNKG	Unknown		e
	XNLRFDSDVGEFRAV	H-2Eβ^k	33– 47	e
	EDENLYEGLNLDDXSMYE	MBI	177–194	e
	XXLYNKGIMGEdSYPY	Cathepsin H	77– 92	e
	SYLDAXVXEQLAT	Fcε-Receptor II	298–310	e
	XXXHFVHQFQPFcyF	H-2Aβ^k	3– 17	e
	QFQPFXYFTNT	H-2Aβ^k	10– 20	e
H-2A^{g7}	KPKATAEQLKTVMDD	Serum albumin	560–574	f
	GHNYVTAIRNQQEG	Transferrin	55– 68	f
	ETTEESLRNYYEQ	hnRNP B1 & A2	31– 43	f
	VVMRDPASKRSRGFGF	hnRNP A2 & B1	51– 66	f
	VVMRDPQTKRSRGFGF	hnRNP A1	44– 59	f
	PKEPEQLRKLFIGGL	hnRNP A1	7– 21	f
	VVYPWTQRYFDSF	β Globin major	33– 45	f

References:
a: Rudensky et al. 1991; b: Rudensky et al. 1992; c: Hunt et al. 1992b; d: Nelson et al. 1992; e: Marrack et al. 1993; f: Reich et al. 1994

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Definition of Specific Peptide Motifs for Four Major HLA-A Alleles[1]

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Allele-specific motifs for the human MHC class I molecules, HLA-A1, A3, A11, and A24 were characterized by three complementary approaches. First, amino acid sequence analysis of acid eluted peptide pools from affinity purified class I molecules defined putative motifs 9 or 10 amino acids in length and bearing critical anchor residues at position 2 and at the COOH-terminal. These motifs were distinct, with the exception of the HLA-A3 and A11 motifs that were very similar to each other. Second, the correctness of these putative motifs was verified by analyzing the binding capacity of polyalanine peptide analogues to purified HLA-A molecules. Several alternative anchor residues that were not obvious from the pooled peptide sequencing analysis were identified. Third, sequences of individual peptides eluted from HLA-A1, A11, and A24 were determined by tandem mass spectrometry. Nonamers were the predominant species, although peptides of 8, 10, 11, and 12 amino acids in length were also identified. These peptides displayed anchor residues predicted by the specific motifs at position 2 and at the COOH-terminal, regardless of peptide length. Synthetic versions of the naturally processed peptides were shown to bind to the appropriate HLA-A alleles with IC_{50} values in the 0.3- to 200-nM range. A rational approach to search Ags with known amino acid sequences for epitopes restricted by some of the most common HLA-A types and of potential clinical importance is now feasible. *Journal of Immunology,* 1994, 152: 3913.

Most CTLs recognize foreign Ag in association with class I molecules in the form of a peptide fragment bound to the MHC class I molecule (1, 2). These antigenic peptides are normally derived by degradation of various Ags endogenously synthesized by the APC and are translocated into a pre-Golgi compartment where they interact with class I heavy chains to facilitate proper folding and association with β_2-microglobulin (3–6). Finally, these peptide-class I complexes are routed to the cell surface where they can be scrutinized by T cells (1, 2, 6).

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Knowledge of the molecular details of the peptide-class I interaction derives from a large body of recent data, among which are the solution of the three-dimensional structure of the class I molecules and the resolution of the structure of the peptide binding groove occupied by naturally processed peptides or single peptide epitopes (7–14). Furthermore, sequence analysis of naturally processed peptides bound to class I either as mixtures (15–18) or as individual peptides (19–22) revealed unique features of class I ligands. Class I-bound peptides are restricted in length, generally of 9 ± 1 residues, and bear key amino acids at defined positions. These features are referred to as peptide motifs (15, 18).

The original studies of Rammensee et al. (15) on murine MHC class I and human HLA-A2.1 suggested that class I peptide motifs are allele specific. Indeed, many subsequent studies by other investigators have reported specific peptide motifs for a variety of human class I alleles including HLA-A68 (23), A3 (24), A11 (25), B53 (26), B27 (19), and B8 (27). In general, for these human class I molecules, the peptides are 8 to 10 residues in length and bear anchor

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residues occupying the second residue position from the NH_2-terminal and at the COOH-terminal position of the peptide.

It is clear that peptide motifs have value in allowing the identification of potential CTL epitopes from Ags of known amino acid sequences (26, 28, 29) but the therapeutic application of this approach to human diseases requires detailed knowledge of peptide motifs for the most common human class I molecules. We have undertaken this study to define peptide motifs for some of the most frequently expressed HLA-A common alleles, i.e., HLA-A1, A3, A11, and A24. We have taken a three-pronged approach, that included: 1) direct amino acid sequencing of naturally processed peptides eluted from affinity-purified class I as mixtures (sequenced by automated Edman degradation); 2) determining the HLA binding capacity of motifs containing polyalanine peptide analogues; and 3) sequencing of individual peptide species derived from naturally processed peptides (sequenced by tandem mass spectrometry). In the paper, "The Role of HLA-A Motifs In Identification of Potential CTL Epitopes In Human Papilloma Virus Type 16 E6 and E7 Proteins," we address the reliability of these peptide motifs to predict potential CTL epitopes by testing a set of overlapping nonamer peptides of the human papilloma virus, type 16 E6/E7 proteins for binding of the various HLA-A alleles mentioned in this study.[3]

Materials and Methods

Cell lines and Abs

EBV-transformed B cell lines homozygous for HLA-A1 (A*0101) (Steinlin, MAT), A3 (A*0301) (H0301, EHM, GM3107), A11 (A*1101) (BVR, WT100), A24 (A*2401) (KT3, TISI), and A2.1 (A*0201) (JY, LG2) were used as sources for the class I isolation. These cell lines were obtained from the ASHI Repository Collection (*Steinlin, H0301, EHM,* LKT3, TISI), the Human Genetic Mutant Repository (BVR, WT100, GM3107), Dr. Gerald Nepom, Virginia Mason Research Center, Seattle, WA (MAT), Dr. Linda Sherman, Scripps Clinic and Research Foundation, La Jolla, CA (JY), and Dr. Joan Gorga, Children's Hospital Pittsburgh, Pittsburgh, PA (LG2).

The following mAbs were used for this study: BB7.2, anti-HLA-A2 (30) (American Type Culture Collection (ATCC)), GAP A3, anti-HLA-A3 (31) (ATCC), W6/32, anti-HLA-A,B,C (32) (ATCC), and B1.23.2, anti-HLA-B,C (33).

Purification of HLA-A

For the purification of HLA class I for peptide elution, the protocol of Slingluff et al. (34) was used to prepare naturally processed peptides for direct sequencing. For HLA-A2 purification, the mAb BB7.2 was used and for HLA-A3, the GAP A3 mAb was used. In the case of A1, A11, and A24, due to the lack of appropriate reagents for affinity purification purposes, the class I molecules were purified by a sequential immunoprecipitation protocol in which the cell lysates were first depleted of HLA-B and -C Ags by repeated passage (generally ~2 to 4 passages were required to effect depletion) over a B1.23.2 column and then the HLA-A molecules were affinity purified by passage of the lysate over a W6/32 column. Protein purity, concentration, and effectiveness of depletion steps were monitored by SDS-PAGE. Naturally processed peptides were eluted from class I by treatment of the immune complexes with 25% acetic acid at 60°C and collected by filtration through a Centricon filter (3000 kDa cutoff) (Amicon, Beverly, MA).

For class I peptide binding assays, the class I molecules were prepared as previously described (35). Briefly, HLA-A was bound to the appropriate mAb affinity matrix and subsequently eluted with base (50 mM diethylamine) containing 1% octyl glucoside. The eluted class I was neutralized and/or dialyzed against PBS containing 1% octyl glucoside and concentrated using an Amicon ultrafiltration unit (YM30 membrane).

Automated Edman degradation sequencing

Acid eluted peptides were separated by HPLC (Gilson Medical Electronic, Inc., Middleton, WI) using a C18 reverse phase column (0.25 × 25 cm) on a Gilson model 117 HPLC and the gradient described by Stone and Williams (36). Peptides eluting in fractions between 7 and 19% and 20 to 45% acetonitrile, respectively, were pooled and lyophilized. The peptide pools were dissolved in a small volume of 0.1% TFA in water and subjected to amino acid sequencing on an ABI model 477A protein sequenator equipped with an on-line phenyl thiohydantoin amino acid analyzer (model 120A) and 610 data analysis software (Applied Biosystems, Inc., Foster City, CA). Cysteine was not modified and thus not detected. PTH tryptophan co-eluted with diphenylurea and in some experiments PTH arginine co-eluted with the major derivative of PTH threonine. Thus, cysteine and tryptophan were not detectable and arginine was detected only in the absence of threonine. In initial experiments, residues showing at least a twofold increase in yield from the previous cycles were considered arbitrarily significant in abundance, following the convention introduced by Falk et al. (15).

To allow the averaging of data from different experiments involving the same HLA type, and thus help establish the significance of the various increases observed, we introduced a more rigorous method of data analysis that allows quantitative, standardized values to be generated. The raw sequenator data was converted to a simple matrix of 10 rows (each representing one Edman degradation cycle) and 16 columns (each representing 1 of the 20 amino acids; W, R, and H were eliminated for technical reasons). The data corresponding to the first row (first cycle) was not considered further because this cycle is usually heavily contaminated by free amino acids. The values of each row were summed to yield a value for total picomoles for that particular cycle. For each row, values for each amino acid were then divided by the corresponding total yield value to determine what fraction of the total signal is attributable to each amino acid at each cycle. By doing so, an absolute frequency table was generated (data not shown). This absolute frequency table allows correction for the declining yields of each cycle.

Starting from the absolute frequency table, a relative frequency table was then generated to allow comparisons among different amino acids. To do so, the data from each column were summed and then averaged. Then, each value was divided next by the average column value to obtain relative frequency values. These values quantitate in a standardized manner increases and decreases per cycle for each of the different 16 amino acid types. Tables generated from data from different experiments can thus be added together to generate average relative frequency values (and their SDs). All SDs can then be averaged to estimate a SD value applicable to the samples from each table. Any particular value exceeding 1.00 by more than two SDs is considered to correspond with a significant increase.

Peptide sequencing by triple quadrupole mass spectrometry

The direct sequence analysis of peptide in mixtures by triple quadrupole mass spectrometry was performed as previously described (20).

Soluble class I binding assay

This was performed as previously described (35). Briefly, indicated amounts of class I were incubated in 0.05% Nonidet P-40/PBS with ~5 nM of radiolabeled peptides in the presence of 1 to 3 μM human β_2M (Scripps Laboratories, San Diego, CA) and a mixture of protease inhibitors (final concentration: 1 mM PMSF, 1.3 mM 1, 10 phenanthroline, 73 μM pepstatin, 8 mM EDTA, and 200 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone). A

[3] Kast, W. M., R. M. P. Brandt, J. Sidney, J.-W. Drijfhout, R. T. Kubo, H. M. Grey, C. J. M. Melief, and A. Sette. 1994. The role of HLA-A motifs in identification of potential CTL epitopes in human papillomavirus type 16 E6 and E7 proteins. *J. Immunol.* 152: *3904.*

panel of synthetic peptides that would be predicted to have high binding affinity for the class I molecules was sought to provide suitable candidates for radiolabeling. Because there was scarce information regarding non-HLA-A2.1-restricted epitopes, we derived "consensus" peptide sequences for putative high affinity specific binders based on the amino acid sequencing data of the naturally processed HLA-A-bound peptides used to derived the basic peptide motifs. These peptides were YLEPAIAKY for A1, AVDLYHFLK for A11, KVFPYALINK for A3, and AYIDNVYKF and AYIDNYNKF for A24. In all cases, a Y residue was inserted into a position to allow radioiodination. Peptides were iodinated by the chloramine T method described by Buus et al. (37). For HLA-A1 and A24, where a Y residue occupies an anchor position, i.e., the COOH-terminal for A1 and position 2 for A24, a second Y was added for radioiodination. This step was necessary because in the case of HLA-A1 a F residue could not be substituted for the COOH-terminal Y and a consensus peptide with Y only at position 9 did not bind after radioiodination, whereas the peptide YLEPAIAKY was efficiently bound after radiolabeling. After 2 days of incubation, free and MHC-bound peptides were separated by TSK gel filtration as previously described (38). Peptide binding was quantified by an inhibition assay.

Peptide synthesis

Synthesis of peptides was performed using an ABI model 430 peptide synthesizer (Applied Biosystems, Inc.) or the Advanced ChemTech MPS350 multiple peptide synthesizer (Advanced ChemTech, Louisville, KY). Peptides were cleaved with hydrogen fluoride and purified by HPLC, as previously described (35). The purity of the peptides was routinely more than 90%. The peptides were quantified by accurately weighing the lyophilized powder and dissolving in DMSO/water.

Results

Definition of HLA-A allele-specific motifs for HLA-A1, A3, A11, and A24

The studies of Rammensee et al. have shown that allele-specific motifs for both murine and human class I alleles can be identified through the direct Edman sequencing of peptide mixtures eluted from affinity-purified class I molecules. These motifs are characterized in terms of peptide length (generally 9 ± 1 amino acid residues) and dominant amino acids that occupy crucial positions in the peptide, i.e., anchor residues.

In humans, HLA-A2.1 is expressed by a large proportion of individuals of various ethnic backgrounds (ranging from 25% in blacks to 54% among Chinese) (39). Besides A2.1, several other HLA-A alleles are expressed among members of different ethnic origins at frequencies of 15% or more (39). We selected from among these HLA-A1, A3, A11, and A24 on the basis of ready availability of homozygous EBV-transformed B cell lines, of appropriate mAb reagents, and of the availability of nucleotide sequence information on the class I alleles. For all of these alleles, the most common subtype was taken as the prototype sequence. We initially sought to determine putative peptide motifs for HLA-A1, A3, A11, and A24 using the approach described by Falk et al. (15). Two or more sequence analyses were performed for each of the HLA-A alleles. Peptide samples were derived from at least two independent homozygous B cell lines bearing the same A allele.

A representative amino acid sequence analysis for HLA-A3 eluted peptides from the cell line EHM corresponding to pooled fractions between 7 and 19% CH_3CN of the HPLC gradient is shown in Table IA. For each position (except the first), residues showing at least a twofold increase in yield from the previous cycle could be considered arbitrarily significant in abundance, following the method of data analysis described by Falk et al. (15). Contamination by free amino acids caused difficulties in obtaining reliable data for the first cycle. At position 2, significant increases in L, V, I, M, and T were seen. Of these, L and V showed more than fivefold increases and significant picomolar yields. At position 3, Y showed a marked increase over the previous cycle. In all of the other positions from 4 to 8, except for position 5, increases in several residues were noted. At position 9, K is increased more than threefold over the previous cycle. The drop in yield after position 10 suggested that most of the peptides were 9 or 10 amino acids in length.

To allow for combination of the results of repeat experiments and to evaluate the significance of the increases observed, we also introduced a more rigorous method of data analysis that yielded quantitative yet standardized values, whereas allowing the averaging of data from different experiments involving the same HLA type (see *Materials and Methods*). The result of this type of analysis for HLA-A3 is shown in Table IB. At position 2, a 2.2-fold increase in valine (V) with lesser increases (1.5- to 1.7-fold) for structurally similar residues leucine (L) and methionine (M) was observed. At position 3, both tyrosine (Y) and aspartic acid (D) showed significant increases in frequency. At position 7, isoleucine (I) was increased. At position 8, asparagine (N) and glutamine (Q) were increased. The most prominent increases were observed at positions 9 and 10 where lysine (K) was increased more than twofold over the expected random yield. Indeed, previously described motifs for other HLA class I molecules indicated the presence of anchor residues at position 2 and at the COOH-terminal (either position 9 or 10) (19, 23–27). Based on these results, and because the increases observed in positions 2 and 9 or 10 were the most prominent, a putative HLA-A3 motif could entail L, V, or M in position 2, a length of 9 or 10 amino acids, with the COOH-terminal position occupied by K.

Using the same approach (Table II), we identified specific motifs for HLA-A1, A11, and A24. A summary of these motifs is presented in Table III. Using this same approach in control experiments, we have also identified an HLA-A2.1 motif, that is identical to that previously identified by Falk et al. (15), that is, anchor residues leucine (L) or methionine (M) were found at position 2 and valine (V) at the COOH-terminal, respectively, for peptides of 9 or 10 residues in length. For all the different alleles, although strong and weak residues, as originally described by Falk et al. (15), could be identified at several nonanchor positions, these have been omitted from Table III because such residues did not appear to be essential for HLA binding (see below).

Table I. *Pool peptide sequencing of peptides eluted from HLA-A3 molecules*

A Cycle	Amino Acid Residues (in pmols)[a]															
	A[b]	D	E	F	G	I	K	L	M	N	P	Q	S	T	V	Y
1	45.84	871.82	3.39	65.48	53.03	13.87	25.7	7.96	1.55	0.00	6.66	5.27	10.85	6.61	16.04	2.11
2	16.10	213.32	3.31	5.66	25.84	29.21	3.66	50.57	6.03	0.00	6.70	6.20	6.51	20.15	88.29	1.74
3	21.17	90.68	3.72	7.70	21.70	5.47	7.76	13.47	3.78	7.60	9.33	3.96	3.69	4.02	5.58	17.16
4	13.91	30.63	10.05	2.46	53.66	3.91	8.80	7.90	1.23	6.79	22.23	5.60	4.62	6.27	6.10	3.28
5	12.37	29.93	4.71	2.26	44.30	5.44	10.93	5.55	1.46	5.66	25.44	5.44	3.32	4.95	9.33	2.63
6	14.74	15.68	4.24	3.49	27.74	12.08	3.64	24.73	2.33	6.22	22.61	6.08	4.14	5.70	14.01	3.14
7	11.86	18.55	2.85	3.26	19.44	25.61	3.25	20.96	2.20	3.77	9.81	3.96	2.39	4.97	11.97	4.60
8	11.65	12.10	5.64	2.26	19.06	5.76	4.675	9.81	3.47	11.22	10.88	8.28	3.36	5.97	6.82	2.86
9	8.66	9.69	2.67	1.58	13.90	2.71	16.36	4.86	1.03	3.35	5.95	3.65	1.75	2.28	3.91	2.00
10	4.53	8.29	1.57	1.39	12.11	1.53	9.31	2.81	0.50	1.74	3.06	2.23	1.45	1.60	1.71	1.21
11	2.67	8.02	1.10	0.79	10.08	1.12	3.74	1.80	0.35	1.21	1.90	1.50	1.01	1.11	1.05	0.87
12	2.02	6.89	0.99	0.64	9.01	1.03	1.43	1.62	0.28	0.97	1.63	1.00	0.93	1.15	0.41	0.67
B Cycle	Average Relative Frequency[c]															
	A[b]	D	E	F	G	I	K	L	M	N	P	Q	S	T	V	Y
2	1.01	0.57	0.64	1.27	0.73	1.28	0.55	1.55	1.77	0.42	0.51	0.70	1.21	1.38	2.22	1.35
3	0.99	2.00	0.74	1.16	0.80	0.57	0.62	0.74	1.05	0.87	0.66	0.64	1.05	0.80	0.56	1.70
4	0.86	1.34	1.22	0.80	1.22	0.64	0.89	0.54	0.66	1.06	1.29	0.90	1.05	0.93	0.72	0.73
5	0.94	0.96	0.98	0.96	1.22	0.96	1.00	0.72	0.94	0.94	1.47	0.99	0.98	0.99	0.87	0.69
6	1.01	0.84	0.87	1.02	0.98	1.40	0.58	1.27	0.82	0.91	1.21	1.00	1.01	1.03	1.06	0.67
7	1.03	0.76	1.05	0.95	0.93	1.65	0.63	1.35	0.99	1.12	0.96	1.27	0.89	0.93	0.99	0.88
8	1.06	0.73	1.26	0.89	0.99	0.80	1.25	0.96	1.09	1.54	1.01	1.61	1.10	1.17	0.81	0.95
9	1.10	0.81	1.25	0.96	1.15	0.70	2.48	0.86	0.68	1.14	0.89	0.89	0.70	0.77	0.76	1.03
10	1.08	0.86	1.13	1.06	1.35	0.65	2.11	0.77	0.49	1.09	0.83	1.17	1.10	0.65	0.65	0.99

[a] Interpretation of data as described by Falk et al. (15) Underlined values are considered significant.
[b] Amino acids in one letter code.
[c] See text for description of method to calculate the average relative frequency values. Average SD = 0.27 ± 0.22.

In the case of HLA-A1, however, it appeared that a third anchor residue might be present at position 3. Thus, for this allele, two motifs were proposed: one with anchor residues at position 2 (threonine (T)) or serine (S)) and at the COOH-terminal (tyrosine (Y)) for peptides of 9 to 10 residues, and the second motif with an anchor at position 3 (aspartic acid (D) or glutamic acid (E)) and at the COOH-terminal (Y)). Alternatively, a motif with anchor positions at 2, 3, and the COOH-terminal was also considered possible.

Interestingly, the HLA-A11 motif, also shown in Table III, was very similar to the one obtained for HLA-A3. More specifically for HLA-A11 the putative motif was defined by the presence of T or V anchor residues at position 2 and K at the COOH-terminal, i.e., positions 9 or 10. These data are in good overall agreement with two recently published reports independently describing putative A3 (24) and A11 (25) motifs.

Finally, for HLA-A24 the motif was characterized for peptides of 9 to 10 residues as having a Y as the anchor residue in position 2 and a phenylalanine (F) or L residue at the COOH-terminal positions.

In summary, the results obtained by analysis of pooled peptides establish putative motifs for four major HLA alleles. Moreover, they also indicate that HLA-A motifs are general distinct, although some alleles, such as A3 and A11, may have very similar or even overlapping motifs.

Validation of HLA-A allele-specific peptide motifs

We have recently established class I peptide binding assays in solution based on the technology developed to analyze class II peptide interactions and on the knowledge derived from the definition of the class I allele-specific peptide motifs (35). Those studies demonstrated the usefulness of quantitative assays in dissecting the exact contribution of different anchor residues and peptide sizes to the overall affinity of class I peptide interaction. Similar binding assays for HLA-A1, A11, A3, and A24 using purified class I molecules and radiolabeled synthetic peptide probes have recently been developed (A. Sette, unpublished observations).

In this study, quantitative peptide binding assays were used to verify the correctness of the motifs derived from the amino acid sequence data from the peptide pools and to determine whether other residues that were not identified from the pooled peptide sequencing data could also serve as anchors. For this approach, peptides were synthesized with specific HLA-A motifs, i.e., anchor residues, embedded in a neutral backbone composed of alanine residues. The use of such neutral polyalanine backbones as applied to the case of class II molecules has been described in detail (40). In some of the peptides, a lysine residue was introduced within the sequence for the purpose of increasing peptide solubility.

Table II. *Average relative frequencies of amino acids from pool peptide sequencing of peptides eluted from HLA-A molecules*

A: HLA-A1[a] Cycle	Average Relative Frequency[b]															
	A	D	E	F	G	I	K	L	M	N	P	Q	S	T	V	Y
2	1.20	0.69	0.45	1.07	0.78	1.21	0.66	0.98	1.02	0.71	0.59	0.42	2.09	2.54	1.33	0.53
3	1.05	2.45	1.83	0.68	0.95	0.80	0.97	0.61	0.68	0.79	0.70	0.63	1.11	1.02	0.71	0.56
4	0.78	1.09	1.22	1.04	0.93	0.95	1.25	0.79	1.14	0.94	1.53	1.08	0.98	0.97	1.08	0.63
5	1.16	0.85	0.88	0.92	1.10	0.95	1.25	0.66	1.00	1.37	1.29	1.14	0.89	0.82	1.08	0.88
6	0.92	0.79	0.81	1.34	1.25	1.03	0.96	0.88	0.94	1.27	1.26	1.11	0.79	0.83	1.00	0.78
7	1.12	0.64	1.02	0.81	0.96	1.13	0.69	1.53	1.02	1.17	1.00	1.28	0.64	0.54	0.96	0.77
8	0.84	0.87	0.96	1.14	1.02	1.15	1.23	1.40	1.28	0.78	0.79	1.02	0.55	0.54	1.07	0.95
9	0.94	0.63	0.82	1.00	1.01	0.78	0.99	1.15	0.93	0.97	0.85	1.32	0.95	0.73	0.76	2.90
10	0.93	0.69	0.88	0.94	1.25	0.77	0.82	0.94	0.59	1.11	1.14	1.22	0.79	0.67	0.69	2.76

B: HLA-A11[a] Cycle	Average Relative Frequency[b]															
	A	D	E	F	G	I	K	L	M	N	P	Q	S	T	V	Y
2	1.27	0.83	0.43	1.00	0.65	0.81	0.52	0.84	0.52	0.20	1.32	0.59	1.22	1.76	1.78	0.92
3	1.11	1.37	0.65	1.71	0.66	1.09	0.49	1.07	2.38	0.68	0.60	0.82	1.05	0.96	1.02	1.33
4	0.93	1.09	1.20	0.93	1.24	0.73	1.22	0.90	0.93	1.30	1.05	0.86	1.03	0.90	0.59	1.07
5	0.91	1.07	1.17	0.88	1.09	0.76	0.97	0.76	1.03	1.18	1.37	0.91	0.91	0.80	0.83	1.06
6	1.01	1.00	0.97	1.01	1.10	1.19	0.80	1.07	1.27	1.05	1.03	0.92	0.89	0.88	0.81	0.93
7	0.91	0.88	0.97	0.90	1.06	1.38	0.87	1.23	0.76	1.09	0.98	1.02	0.91	0.82	1.07	0.84
8	0.96	0.87	1.33	0.72	1.11	1.06	0.99	1.07	0.79	1.24	0.86	1.50	1.00	0.96	0.97	0.85
9	0.90	0.90	1.28	0.84	1.10	0.98	2.14	1.05	0.33	1.25	0.77	1.39	0.99	0.92	0.93	0.99
10	0.83	1.01	1.32	0.83	1.14	0.83	2.49	1.04	0.47	1.11	0.75	1.50	1.04	1.03	0.75	1.31

C: HLA-A24[a] Cycle	Average Relative Frequency[b]															
	A	D	E	F	G	I	K	L	M	N	P	Q	S	T	V	Y
2	0.71	0.67	0.95	0.78	0.48	0.24	0.25	0.30	0.43	0.10	1.37	0.30	0.59	0.23	0.35	5.58
3	0.76	0.93	0.60	0.82	1.03	1.58	0.57	1.25	1.96	0.69	1.44	1.11	0.79	0.91	0.93	0.47
4	0.70	1.91	1.77	0.48	1.64	0.47	1.66	0.86	0.62	0.99	1.56	0.94	1.07	1.03	0.54	0.32
5	0.84	1.13	0.76	0.51	1.28	1.30	1.50	0.94	1.92	1.61	0.89	1.18	0.96	1.02	1.44	0.24
6	0.93	0.72	0.53	1.36	0.95	1.29	0.78	1.22	1.09	0.79	0.98	0.90	0.70	1.23	1.46	0.30
7	1.16	1.03	1.45	0.73	0.87	1.22	0.79	0.73	0.92	1.72	0.80	1.31	1.00	1.22	1.69	0.35
8	1.54	0.87	1.54	0.61	0.94	0.62	1.55	0.90	0.58	1.45	0.54	1.53	1.81	1.43	1.05	0.51
9	1.36	0.74	0.77	2.71	0.81	1.27	0.89	1.82	0.48	0.65	0.41	0.74	1.08	0.92	0.53	0.22
10	1.75	1.03	0.80	1.98	1.43	0.89	0.85	1.27	0.40	0.64	0.51	0.80	1.02	1.22	0.45	0.32

D: HLA-A2.1[a] Cycle	Average Relative Frequency[b]															
	A	D	E	F	G	I	K	L	M	N	P	Q	S	T	V	Y
2	0.65	0.84	0.38	0.76	0.84	1.06	0.63	2.66	1.93	0.39	0.54	0.60	1.13	0.62	0.78	0.28
3	1.25	1.11	0.59	1.46	0.96	1.20	0.65	1.11	1.91	0.76	0.78	0.84	1.50	0.90	0.69	1.41
4	0.85	1.70	1.73	0.69	1.29	0.53	0.89	0.45	0.62	1.17	1.44	0.92	1.33	0.94	0.60	0.65
5	0.95	1.03	1.10	1.16	1.22	0.93	1.66	0.57	0.71	1.28	1.15	0.95	0.87	0.95	0.79	1.32
6	0.77	0.83	0.82	1.00	0.89	1.49	1.09	1.00	0.68	1.08	1.09	0.90	0.77	1.21	1.38	0.78
7	1.21	0.82	1.05	1.07	0.78	1.15	0.89	0.69	0.88	1.07	1.30	1.16	0.71	1.07	1.24	1.34
8	1.16	0.84	1.45	1.09	1.05	0.76	1.35	0.59	0.54	1.28	0.87	1.63	0.92	1.60	0.84	1.21
9	1.15	0.82	0.87	0.78	0.98	0.88	0.82	0.92	0.73	0.96	0.81	1.00	0.77	0.71	1.69	1.00
10	1.25	1.19	0.88	0.73	1.48	0.54	0.87	0.77	0.22	0.42	1.01	1.00	0.58	0.57	1.27	0.79

[a] Average SD for HLA-A1, 0.26 ± 0.2; HLA-A1, 0.24 ± 0.22; HLA-A24, 0.23 ± 0.18; and HLA-A2.1, 0.25 ± 0.18.
[b] See text for description of method to calculate the average relative frequency values. Underlined values are considered significant.

The results of the binding analysis of these poly A peptides are shown in Table IV. In all cases, the presence of the specific HLA-A motif anchor residues was sufficient to permit binding of the peptide to the relevant HLA-A allele product. Estimated K_ds ranged from 3 to 125 nM for the nonamers and from 4 to 148 nM for the decamers. In most cases, the binding of the peptide analogue was highly specific in that no binding was detected to the other alleles, e.g., AADAAAAA(A)Y for A1, ALAKAAAA(A)V for A2.1, and AYAKAAAA(A)F for A24. In the case of A3 and A11 it was predicted that because the motifs for these two alleles are remarkably similar, the corresponding analogues would cross react extensively. The data in Table IV show that this was indeed the case. Some of the HLA-A1 motif peptides also cross-reacted, albeit more weakly, with A3 and A11.

In the case of HLA-A1, various 9 mer peptides were synthesized to reflect various possible combinations of the

Table III. *Summary of HLA-A allele-specific motifs*

Allele	Position 1	2	3	4	5	6	7	8	9/10
A1	•	T,S	•	•	•	•	•	•	Y
A1	•	•	D,E	•	•	•	•	•	Y
A2.1	•	L,M	•	•	•	•	•	•	V
A3	•	V,L,M	•	•	•	•	•	•	K
A11	•	T,V	•	•	•	•	•	•	K
A24	•	Y	•	•	•	•	•	•	F,L

anchor residues defined by pooled peptide sequencing. All peptides had a common COOH-terminal Y anchor residue but one carried the putative anchor residue T at position 2, whereas the second had the putative anchor residue D at position 3 and the third had both putative anchor residues at positions 2 and 3 (T and D, respectively). The corresponding 10 mer analogues were also synthesized. It was found that all poly-A analogues carrying putative A1 anchors at either position 2 or position 3 bound well in the 15- to 60-nM range. When analogues carrying analogues at both position 2 and 3 were tested, it was found that they bound even better in the 2- to 4-nM range.

In summary, the results of the analysis of the poly-A-based analogues confirm the putative HLA motifs proposed on the basis of the pooled peptide sequencing data.

Definition of the permissiveness of major anchor positions in HLA-A binding

To address the issue of whether other chemically related amino acids might also be tolerated in the crucial anchor positions, additional analogues of some of the HLA binders listed in Table IV were synthesized by varying the residues present in positions 2, 3, or 9. The peptides were then evaluated for binding to the appropriate HLA-A alleles.

The analysis of the requirements for HLA-A3 binding following this approach is shown in Figure 1*A*. With position 9 fixed with a lysine residue, it was seen that peptides bearing M, V, I, S, A, T, and F at position 2 all bound HLA-A3 with affinities that were within 10-fold of the parent peptide analogue, ALAAAAAAK. Peptides bearing C, G, and D showed lower affinities of binding (10- to 100-fold decreases), whereas substitution with K, Y, or P resulted in peptides that did not bind to HLA-A3 at all. When position 2 was fixed with leucine, it was shown that substitution at position 9 with R or Y yielded peptide analogues that bound well to HLA-A3. Weak binding was seen with the alanine substituted analogue. All other substitutions tested, e.g., Q, S, T, N, and E, resulted in the complete loss of binding capacity. These results indicate that for HLA-A3 alternative amino acids (i.e., I, S, A, F, T) that were not obvious in the pooled peptide sequencing analysis can occupy the anchor positions in peptides capable of binding to HLA-A3 and R, Y, or perhaps A can occupy the COOH-terminal anchor position.

Table IV. *HLA-A motif anchor residues in polyalanine nonamers and decamers*

Sequence	Motif	Binding Capacity (IC_{50} nM) A1	A2.1	A3	A11	A24
ATAKAAAAY	A1	15	—[a]	329	77	—
AADKAAAAY	A1	50	—	—	—	—
ATDKAAAAY	A1	3	—	9,250	840	—
ALAKAAAAV	A2.1	—	125	—	—	—
ALAAAAAAK	A3	—	—	85	33	—
ATAAAAAAK	A11	—	—	59	40	—
AYAKAAAAF	A24	—	—	—	—	115
ATAKAAAAAY	A1	58	—	1,100	1,030	—
AADKAAAAAY	A1	45	—	—	—	—
ATDKAAAAAY	A1	4	—	10,000	4,533	—
ALAKAAAAAV	A2.1	—	1,400	—	—	—
ALAAAAAAAK	A3	—	—	148	49	—
ATAAAAAAAK	A11	—	—	216	88	—
AYAKAAAAAF	A24	—	—	—	—	35

[a] A dash indicates an IC_{50}% greater than 10,000 nM.

The same set of polyalanine analogues tested above for HLA-A3 binding were also analyzed for HLA-A11 binding capacity. As shown in Figure 1*B*, alternative amino acids in the anchor position 2 included M, A, V, I, T, S, G, and N. The peptide with M at position 2 bound to A11 with a slightly greater affinity than the parental polyalanine analogue. Peptides with C, F, or D at position 2 bound less well, whereas peptides with K, Y, or P at this position bound poorly at best. At the COOH-terminal anchor position, with position 2 fixed with a leucine residue, only the COOH-terminal K analogue bound well. The arginine substituted peptide showed only weak binding relative to the parental peptide. Thus, although HLA-A3 and A11 have similar peptide motifs, some differences in the tolerated amino acid substitutions in the anchor positions could be detected using the polyalanine peptide analogues.

In the case of HLA-A24 only a phenylalanine residue could serve as an alternative anchor residue at position 2 with M yielding a weaker binding peptide (Fig. 1*C*). At the COOH-terminal position, tryptophan, leucine, and isoleucine were found to be acceptable anchor residues. Indeed, the presence of a leucine at the COOH-terminal position was also deduced from the analysis of the pooled peptide sequencing data. In the case of HLA-A1, the data in the previous section demonstrated that position 2 and position 3 anchor residues might act independently of each other, but both in the context of the same position 9 anchor residue. Thus, to evaluate the requirements for HLA-A1 binding, we synthesized analogues containing anchor residues at each of the three potential anchor positions. Consistent with the predictions of the sequencing data, in position 2, T or S appear to be capable to serve as anchor residues (Fig. 1*D*). In addition, M was also identified as an alternative anchor residue at this position. In position 3, D and to a somewhat lesser extent E could also serve as

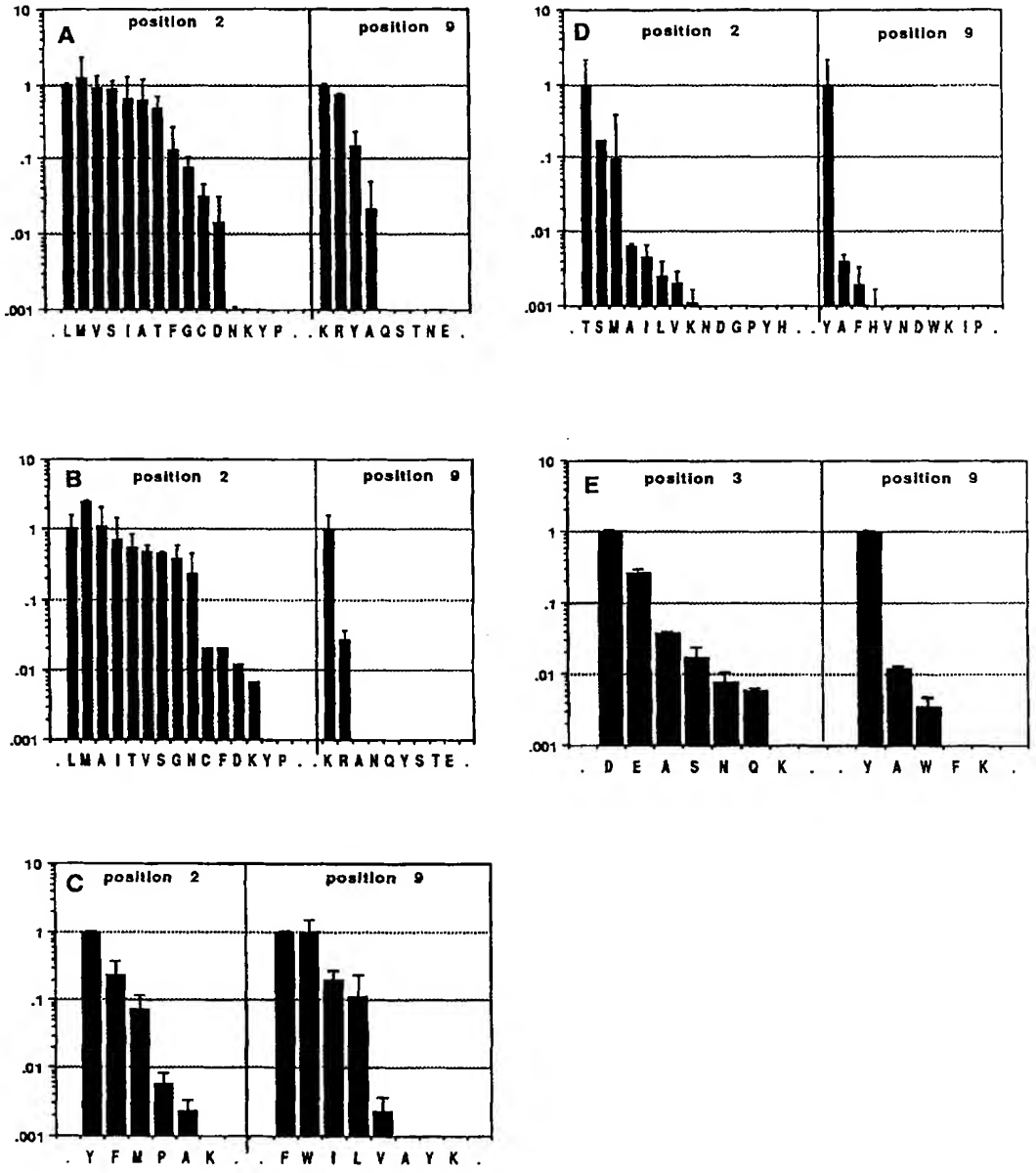

FIGURE 1. Binding of polyalanine analogues to HLA-A molecules. Single amino acid substitutions of the parental polyalanine nonamer peptides as indicated below were tested for binding to purified HLA-A3 (*A*), HLA-A11 (*B*), HLA-A24 (*C*), and HLA-A1 (*D* and *E*). The parental polyalanine nonamer peptide is ALAAAAAAK for HLA-A3 and HLA-A11, AYAKAAAAF for HLA-A24, and ATAKAAAAY (*D*) or AADKAAAAY (*E*) for HLA-A1. The indicated residues along the horizontal axis of each panel were introduced at the anchor positions 2 and 9 (*A* to *D*) or at anchor positions 3 and 9 (*E*). The relative binding capacity is indicated in the vertical axis and the binding of each peptide analogues is presented relative to the parental unsubstituted peptides in each panel.

anchor residues. In position 9, no residue that could replace the crucial tyrosine anchor residue was identified, and even the chemically related phenylalanine residue yielded a peptide analogue with only marginal binding capacity for HLA-A1.

In summary, the data shown in these sections validate the peptide motifs derived from the pooled peptide sequencing data. Furthermore, the results obtained with the polyalanine peptide analogues underline that the binding pockets of different class I molecules differ drastically in

Table V. *Expanded HLA-A allele-specific motifs*

Allele	Position 2	Position 3	Position 9 or 10
A1	T, S, M[a]	D, E (A, S)	Y
A3	L, M, I, V, S, A, T, F, (C, D, G)[b]		K, R, Y, (A) [H, F][c]
A11	L, M, I, V, S, A, T, G, N, (C, D, F)		K, (R) [H]
A24	Y, F (M) [W]		F, L, I, W [M]

[a] Amino acids are considered preferred residues when the binding of polyalanine analogues bearing such residues in the anchor position to the respective HLA-A allele is within 10-fold of the parental peptide.
[b] Amino acids listed in parentheses are residues that are tolerated, i.e., analogues carrying these substitutions bind but less strongly (10- to 100-fold decrease in binding affinity).
[c] Amino acids listed in brackets are speculated to bind based on chemical similarity.

Table VI. *Naturally processed peptides from HLA-A1*

Sequence/Data Base Match	Peptide Length	Source Protein (reference)	HLA Binding (nM)
YTDYGGLIFNSY	12	Cytochrome *c* oxidase (41)	1.9
YLDDPDLKY	9	Cytosine methyl transferase (42)	2.8
NRKLLDIA	8	Corticotropin release factor (43)	—[a]
IADMGHLKY	9	Prolif. cell nucl. factor (44)	3.2
YTSDYFISY	9	Transforming prot. (ets-1) (45)	5.3
STDHIPILY	9	Fructose-6-p-amino transfer. (46)	1.7
YTAVVPLVY	9	Human J chain 102-110 (47)	0.30
DSDGSFFLY	9	Ig gamma-4, 279–287 (48)	0.60
DAKIRIFDL	9	Laminin receptor frag. (49)	—
ATDFKFAMY	9	Cyclin prot. D type (50)	0.74
GTDEXRNXY[b]	9		3.0
ETDXXXDRSEY	11		10
FTDVNSXXRY	10		1.4
YTNPQFNVY	9		25
VXDPYNXKY	9		11
VADKVHXMY	9		12
ETXXPDWSY	9		33

[a] Dash indicates greater than 50,000 nM.
[b] X represents Leu/Ile.

the degree of permissiveness. In some cases, many different amino acids can occupy the same anchor position (e.g., position 2 in A3 and A11), whereas in other instances (e.g., A1 and A24) only a few alternative amino acids are allowed.

On the basis of the results presented above, we also propose a refinement of the motifs for the different HLA-A alleles to include alternative anchor residues that were not predicted from the pooled sequencing data. These expanded HLA-A motifs are shown in Table V.

Identification of peptide sequences by tandem mass spectrometry and binding analysis of the naturally processed peptides

We sought additional information about the HLA-A-specific motifs by tandem mass spectrometry sequencing of individual peptides eluted from either for HLA-A1 (Table VI), HLA-A11 (Table VII), or HLA-A24 (Table VIII).

In the case of A1 it is notable that, in general, nonamer peptide sequences predominated (13 of 17), although peptides of 8, 10, 11, or 12 residues were also identified. A tyrosine (Y) at the COOH-terminal position was observed for 15 of 17 sequences identified. At position 2, T was most frequently found (10 of 17), although L and A were seen in addition to S. At residue 3, D was found very frequently, and interestingly, in many cases, D was also accompanied by a T in position 2. Overall, 15 of 17 peptide sequences carried the HLA-A1 motifs based on T or S in position 2 and/or a D in position 3, both with a COOH-terminal Y residue. Two additional sequences did not carry a COOH-terminal Y.

Synthetic peptides corresponding to these naturally processed peptides were prepared and tested for binding to HLA-A1. All of the motif-bearing peptides bound well, with IC_{50} values in the range of 0.3 to 33 nM. By contrast, the two peptides that did not carry an A1 motif did not bind at all. Thus, it is probable that these sequences were not derived from HLA-A1 bound peptides but more likely were derived from peptides associated with small amounts of HLA-B or HLA-C allele products that might have contaminated the HLA-A1 sample. In this context, it is interesting to note that the NRKLLDIA peptide bears a HLA-B8 motif and the EBV cell line, Steinlin, from which HLA-A1 was purified expresses HLA-A1 and B8 (58). It is also possible that some of the nonmotif-containing peptides could have originated from protein contaminates of the affinity-purified class I preparations that were generally estimated to be 70 to 90% pure.

All of the eight A11 peptide (Table VII) sequences identified terminated with a K residue. At position 2, T, S, A, V, and L/I were found. All of these amino acids were consistent with the expanded HLA-A11 motif described above. Nonamer peptides were identified with the greatest frequency (6 of 9), although 10 to 12 amino acid residue peptides were also sequenced. Synthetic peptides corresponding to these sequences bound very well to HLA-A11, with IC_{50} values in the 2- to 55-nM range.

Because of the similarity between the HLA-A3 and A11 motifs, and because of the cross-reactivities observed with the polyalanine analogues, the set of A11 naturally processed peptides was also tested for A3 binding capacity. It was found that the A3.2 binding capacity of the A11-derived peptides varied dramatically, with some peptides binding equally well to A3 and A11 and others cross-reacting only very weakly.

Finally, for HLA-A24 (Table VIII) the peptide sequences identified also confirm the peptide motif derived from the pool peptide sequencing data. In all cases, a Y was found at position 2 and at the COOH-terminal positions L/I and F were identified. Binding constants for the synthetic HLA-A24 peptides were in the 4- to 100-nM range (IC_{50} values).

Table VII. *Naturally processed peptides from HLA-A11*

Sequence/Data Base Match	Peptide Length	Source Protein (reference)	Binding to HLA-A11 (nM)	Binding to HLA-A3 (nM)
RTQNVLGEK	9	Ribosomal protein, S3 (51)	31	155
SVLNLVIVK	9	Ribosomal protein, S6 (52)	6.94	128
ASFDKAKLK	9	Thymosin B-10 (53)	15.12	42
KVVNPLFEK	9	Ribosomal protein L7A-Rat (54)	8.1	85
ATAGDGXXELRK[a]	12	Rat prohibitin (55)	18	2,400
AAMXDTVVFK	10		8	256
GTMTTSXYK	9		4.2	3.4
RVEQAVESMVK	11		54	1,170

[a] X represents Leu/Ile.

Table VIII. *Naturally processed peptides from HLA-A24*

Sequence/Data Base Match	Peptide Length	Source Protein (reference)	HLA Binding (nM)
KYPENFFLL	9	Phospho prot. phosphatase-1, (91–99) (56)	4.8
YYEEQHPEL	9	Unk. prot. act. K^+ T cell (57)	100
AYVHMVTHF	9		23
VYXKHPVSX[a]	9		4.2

[a] X represents Leu/Ile.

Discussion

The goal of this study was to define specific peptide binding motifs for several HLA-A alleles that are expressed at high frequencies among different ethnic populations by the combined use of amino acid sequencing of naturally processed peptides bound to MHC class I either as mixtures or as individual peptides and the analysis of synthetic peptide analogues in quantitative class I peptide binding assays.

The HLA-A2.1 allele is the most frequently expressed class I allele among the different ethnic populations (39), and a considerable amount of information on its crystal structure (7–14) and on the motif of peptides bound to HLA-A2.1 has been published (15). However, because a significant fraction of the population does not express HLA-A2.1, the inclusion of four additional HLA-A alleles (HLA-A1, A3, A11, and A24) would allow coverage of a significant proportion of many ethnic populations (39). More complete coverage of all ethnic populations will require that several other HLA-A alleles be evaluated and/or that HLA-B and HLA-C alleles of moderate to high frequency of occurrence be considered.

Using the basic approach introduced by Rammensee et al. (15), putative class I motifs for the selected HLA-A alleles were defined by amino acid sequencing of pooled peptide mixtures eluted from affinity-purified class I molecules. The motifs determined by this approach are presented in Table II. The HLA-A3 and A11 motifs reported herein are in good agreement with those independently identified by DeBrino et al. (24) and Zhang et al. (25), respectively.

The second approach to defining motifs was based on the analysis of polyalanine analogues of motif containing peptides. Using this procedure, we have been able to validate the putative motifs identified by peptide pool sequencing and to expand them through the identification of amino acids that can serve as alternative anchor residues (see Table IV). Interestingly, in the case of HLA-A3 and A11 a rather large array of amino acids is tolerated in position 2 of the polyalanine analogues, suggesting that the B pockets of these class I alleles are somewhat accommodating. In contrast, in the case of HLA-A24, only a few position 2 anchor residue substitutions appear to be tolerated. In fact, both alternative anchor residues tend to be aromatic in nature. In the case of the COOH-terminal anchor position most of the alternative anchors are chemically related residues, either charged or hydrophobic. In the case of A3, our amino acid sequencing analysis identified only K as a dominant residue but clearly R-terminated peptides with the appropriate position 2 anchor residue also bound to A3. This may be expected due to the similar chemical nature of this amino acid. In addition, peptides that bear a Y at the COOH-terminal might also bind to A3. On the basis of the amino acid sequencing data, this was not obvious. DiBrino et al. (24) have shown, however, that Y and K are common COOH-terminal anchor residues for peptide bounds to HLA-A3 (24). The binding of peptides terminating with Y to A3 was, therefore, not unanticipated.

The determination of the amino acid sequences of individual naturally processed peptides is a third approach to defining and validating allele-specific peptide motifs. From the data presented in Tables VI to VIII, it is apparent that the motifs identified by pool sequencing were confirmed by the sequencing of individual peptide sequences. In most instances, the preferred residues identified by the polyalanine analogue analysis were found at anchor positions and the peptides identified were generally nonamers, although a few 8, 10, 11, and 12 amino acid peptides were also identified. Similar results have been reported for peptides sequenced from HLA-A2.1 by Henderson et al. (21) and from HLA-A2, HLA-A68, and HLA-B40 molecules by Harris et al. (59). Furthermore, when the synthetic peptides corresponding to naturally processed peptides identified by tandem mass spectroscopy were tested for binding, it was found that 26 of 29 (89%) bound with an estimated $K_d \leq 50$ nM and 2 of 29 (6%) in the 50- to 500-nM range. Similar results have been obtained when the affinity of A2.1 naturally processed peptides (A. Sette et al., manuscript in preparation) or of known T cell epitopes (A. Sette et al., manuscript in preparation) has been analyzed. These data are important in that they represent the first quantitative estimates of the HLA binding affinity of biologically relevant peptide epitopes.

Table IX. *Polymorphism in different HLA pockets*

A Allele	B Pocket[a] (All 10 Position Residues Associated with B Pocket Are Listed)									
	7	9	24	34	45	63	66	67	70	99
A1	Y	F	A	V	M	E	N	V	H	Y
A2.1	Y	F	A	V	M	E	K	V	H	Y
A3	Y	F	A	V	M	E	N	V	Q	Y
A11	Y	Y	A	V	M	E	N	V	Q	Y
A24	Y	S	A	V	M	E	K	V	H	F
Aw68	Y	Y	A	V	M	N	N	V	H	Y

B Allele	F Pocket[a] (Only Polymorphic Positions Are Listed)[b]		
	77	80	116
A1	N	T	D
A2.1	D	T	Y
A3	D	T	D
A11	D	T	D
A24	K	I	Y
Aw68	D	T	D

[a] Sequences from Kabat et al. (59).
[b] Nonpolymorphic residues at 84, 133, 143, 146, and 147.

It is also interesting to analyze the results obtained in the context of the known structure of other class I molecules. Based on the x-ray structure of peptide bound to HLA-A2.1, Aw68, and B27, the side chains of the amino acid in position 2 interacts with residues that constitute the B pocket of the class I peptide binding groove, whereas the COOH-terminal anchor residue is bound by the F pocket (9, 10, 23, 60–63). In Table IXA, the amino acid residues that are thought to make up the B pocket are listed for the six HLA-A alleles for which peptide motifs have been reported (this study, A2.1 (15), A3 (24), A11 (25), Aw68 (23)). Polymorphisms are seen at positions 9, 63, 66, 70, and 99, whereas positions 7, 24, 34, 45, and 67 are invariant. In the motifs of HLA-A2.1, A3, A11, and Aw68 position 2 is generally occupied by a hydrophobic amino acid. The structural basis for B pocket specificity, however, is not easily explained on the basis of a simple comparison of the B pocket sequences. The B pocket residues of HLA-A1 differ from A2.1 and A3 each by a single residue, and yet for HLA-A1 the anchor residues preferably found at position 2 is T or S. By contrast, both Aw68 and A2.1 in position 2 favor V and yet the differences in residues of the B pocket compared with A2.1 are more numerous (three of five polymorphic differences). Better appreciation of the relationships between the B pocket residues and the position 2 anchor residue is realized from the analysis of the coordinates from the three-dimensional structures of HLA-A2.1, Aw68, and B27.5 as performed by Guo et al. (64). Their study indicates that the specificity differences between HLA-A2.1 and Aw68 appears to be due to a change in the conformation of residue 67 (V) and on the sequence difference at residue 9. Additional insights into the basis for peptide binding selectivity may derive from the identification of additional motifs for other HLA alleles and from x-ray structures of other HLA molecules or molecular modeling studies (65).

A listing of the polymorphic residues in the F pocket of the peptide binding groove is also shown for the same HLA alleles in Table IXB. The F pocket residues for HLA-A3, A11, and Aw68 are identical. These three HLA-A alleles differ by a single residue from HLA-A1 (D→N at 77) and from HLA-A2.1 (D→Y at 116). In the case of HLA-A1, Y is the preferred residue at the COOH-terminal, whereas in A2.1 the COOH-terminal position favors the small hydrophobic residue, V. The presence of two aspartic acid residues among the three polymorphic residues in the F pocket of HLA-A3, A11 and Aw68 would explain the preference for a positively charged residue at the COOH-terminal of the peptide motif. The F pocket of A24 differs in all three polymorphic positions from the other HLA-A alleles listed. Thus, in the case of the F pocket it is easy to understand the use of similar anchor residues in the HLA-A3, A11 and Aw68 motifs. In analyzing the available HLA-A allele α-chain sequences (66) it appears that there are several alleles that share identical F pocket residues, e.g., A2, A*6901 and A*6802; A*0301, A*1101, A28, A*6001, A*3301, A*3101 and A*6801; A*0101, A*2601 and A*2901; and A*2501 and A*3201. If alleles with identical F pockets will, in general, use similar COOH-terminal anchors, then a rather limited variability at the COOH-terminal ends of HLA-A allele motifs would be expected. A24 stands uniquely different among the HLA-A alleles in the F pocket residues.

The peptide motifs for the HLA type analyzed in this study are generally distinct from each other and they are also different from the motifs that have been described for other HLA types such as A2.1 (P_2 L, M; P9, V) (15) and Aw68 (P_2, V; P9, R) (23). Previous studies of Carreno et al. (67) concluded that HLA-A3 and HLA-B27 have peptide binding sites that are functionally distinct. Our results support that conclusion and further extend the observation among HLA-A alleles.

One notable exception is the peptide motifs for HLA-A3 and A11, which are very similar to each other. Despite the similarity of the A3 and A11 peptide motifs, the actual A3 and A11 binding capacity of motif-bearing peptides does, however, vary considerably. Some peptides bind equally well to HLA-A3 and HLA-A11, whereas other peptides bind preferentially to one of the two alleles as illustrated by the results obtained when HLA-A11-derived naturally processed peptides were tested for binding to A3. These results suggest that factors other than the nature of the crucial anchor residues may influence peptide binding to HLA molecules (35). Indeed, we have recently demonstrated a prominent role for secondary anchor residues in the case of HLA-A2.1 molecules. We are now in the process of analyzing whether positions other than the

position 2 and the C termini might influence peptide binding to other HLA alleles.

Finally, it has been noted that several HLA-A2.1-restricted epitopes identified by CTL recognition did not bear the A2.1 motif defined by pool sequencing. Moreover, some of the naturally processed A2.1-bound peptides that have been sequenced did not show this canonical A2.1 motif. This raised some concern for the reliability of the peptide motifs to predict CTL epitopes. This question is addressed in the accompanying paper by testing a set of overlapping nonamer peptides of the HPV 16 E6/E7 for binding to various HLA-A alleles (W. M. Kast et al., manuscript submitted)[3]. It was found that the vast majority of peptides binding to a given allele with high and intermediate affinity bear the corresponding HLA motifs. Thus, knowledge of the specific motifs for the most frequent HLA alleles and the availability of quantitative class I peptide binding assays will greatly aid in the search for potential CTL epitopes with clinical relevance.

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Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules

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The crystal structures of major histocompatibility complex (MHC) molecules contain a groove occupied by heterogeneous material thought to represent peptides central to immune recognition, although until now relatively little characterization of the peptides has been possible. Exact information about the contents of MHC grooves is now provided. Moreover, each MHC class I allele has its individual rules to which peptides presented in the groove adhere.

T LYMPHOCYTES recognize their antigens in context of MHC-encoded molecules, a phenomenon called MHC restriction[1-5]. Crystallography of human MHC class I molecules, HLA-A2 and Aw68, revealed a groove made up by the $\alpha 1$ and $\alpha 2$ domains of heavy chains[3,6]. This groove is believed to be the binding site for antigenic peptides, as both crystals contain peptide-sized structures not compatible with the MHC sequences located at that groove[6]. T cells can recognize synthetic peptides loaded on MHC class I molecules, and MHC-associated peptides representing T-cell epitopes have been extracted from normal or virus-infected cells[2,4,5,7,8]. Similarly, the antigens recognized by MHC class II-restricted T cells can be mimicked by artificial peptides[9], and MHC-associated antigenic peptides have been eluted from MHC class II molecules[10]. By virtue of their position in the middle of trimolecular complexes made up of T-cell receptor, peptide and MHC molecule[11], T-cell epitopes are literally central to the specific immune system and the so far unknown rules defining them should be central to understanding it[12-15].

Comparison of the first naturally processed H-2K^d-restricted T-cell epitope[4] with several peptides that contain other K^d-restricted epitopes revealed some common features not found in non-K^d-restricted epitopes[47]. Assuming that all naturally processed K^d-restricted epitopes are nonapeptides, all those peptides containing K^d-restricted epitopes could be aligned with a Tyr residue at the second position and an amino-acid residue with a side-chain methyl group (Val, Ile, Thr, Ala or Leu) at the last position, suggesting a K^d-specific peptide motif. (Tyr is important in K^d-restricted peptides, and some allele-specificity of T-cell epitopes has been noticed before[12-16].) Sequencing the self-peptide blends eluted from purified K^d molecules should reveal this K^d-specific motif. If so, sequencing of peptide mixtures from other MHC class I molecules should reveal the respective motifs if they exist.

Elution of peptides from K^d molecules

K^d molecules were immunoprecipitated from detergent extracts of P815 tumour cells (H-2^d). Bound peptides were dissociated from bead-associated K^d molecules by acid treatment[7] and separated by reversed-phase HPLC (Fig. 1*a*, *b*). Precipitation from influenza-infected cells (not shown) gave a K^d-restricted influenza epitope in fraction 24, indicating that the method can isolate MHC-bound peptides. Heterogeneous material elutes in low amounts between fractions 20 and 28 (Fig. 1*a*, *b*), covering the range of many MHC class I-restricted T-cell epitopes with this HPLC-gradient (refs 4, 7, 8, and O.R. *et al.*, manuscript submitted).

K^d-restricted peptide motif

Fractions 20–28 were pooled both from the K^d-derived batch and from a mock precipitate, and both batches sequenced automatically using the Edman degradation method (Table 1). This method involves the sequential derivatization and removal of amino acids from the N terminus, each of which is identified chromatographically. Because it is unusual to sequence complex mixtures of peptides, we have presented the raw data from the sequencer. Table 1*a* and *b* shows the results from two sequencing attempts for K^d-eluted peptides. Table 1*c* shows the sequencing result of a mock elution with D^b-specific antibodies on P815 lysates. The K^d-eluted peptides have a distinct amino-acid residue pattern for each position from 1 to 9, whereas the mock-eluted material shows a uniform pattern of residues throughout, with a decrease of the absolute amount of each residue with every cycle. Thus, for the K^d-eluted peptides, only residues showing more than 50% increase in the absolute amount compared with the previous or the pre-previous cycle were arbitrarily considered significant and are underlined. The first position is difficult to judge; there is no previous cycle, and all free amino acids present in the HPLC pool are detected in this position. For the second position, the only residue whose frequency is clearly increased by comparison with the previous cycle is Tyr, in both attempts (Table 1*a*, *b*), for example, 60.9 to 875.6 pmol. The only other residue that shows an increase, however marginal, is Phe, which has a side chain similar to that of Tyr. This confirms our premises resulting from comparing the natural K^d-restricted influenza epitope TYQRTRALV (single-letter amino-acid code) with other K^d-restricted peptides, with regard to the Tyr at position 2. By contrast to the second position, there is no single amino-acid residue standing out in the following positions up to 8, although up to 14 different residues are detected in the individual positions. At position 9, the residues detected are Ile and Leu. This again agrees with our premises. There is no signal increase at position 10, indicating that most K^d-bound self-peptides are no longer than 9 residues. The natural K^d-restricted influenza peptide is also a nonapeptide[4]. The consensus sequence pattern indicated by these data is shown in Table 1*d*. Most evident are Tyr at position 2 and Ile or Leu at 9, whereas at all other positions a larger, but distinct set of residues was found. A comparison of this motif with peptide sequences containing K^d-restricted epitopes indicates that, if aligned by their Tyr residues, most fit quite well with the consensus K^d-restricted nonamer motif (Table 1*d*).

Sequence of a prominent self-peptide

The peak marked by an arrow in fraction 29 of Fig. 1*b* and the corresponding fraction of the mock precipitation were rechromatographed giving higher resolution (Fig. 1*c*). The sharp specific peak was determined to be SYFPEITHI by direct

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TABLE 1 Sequencing of the self-peptide mixture eluted from immunoprecipitated K^d molecules

(*a*) Experiment 1

Cycle	A Ala	R Arg	N Asn	D Asp	E Glu	Q Gln	G Gly	H His	I Ile	L Leu	K Lys	M Met	F Phe	P Pro	S Ser	T Thr	Y Tyr	V Val
	Amino-acid residues (in pmol)																	
1	172.8	46.1	44.9	13.6	73.5	317.8	171.6	3.2	73.1	66.5	231.2	28.0	35.3	56.7	145.2	73.3	60.9	130.9
2	25.6	14.1	10.1	7.7	18.7	71.9	71.9	1.2	28.4	22.6	13.9	11.1	97.7	14.8	14.6	9.3	875.6	18.8
3	88.7	26.7	51.5	10.0	23.1	86.8	62.5	2.9	183.2	308.7	71.6	25.6	41.5	13.5	24.0	22.0	66.1	150.2
4	158.5	14.2	31.9	17.9	53.3	44.8	85.2	6.7	32.1	36.6	29.5	9.2	5.8	226.9	26.2	19.9	14.7	41.5
5	139.0	30.1	42.2	22.9	15.1	44.1	154.5	1.8	59.3	86.6	10.2	50.8	2.6	87.8	64.2	47.6	8.8	104.2
6	116.5	29.2	42.6	13.0	10.6	38.3	139.1	8.5	90.1	99.9	194.5	69.7	27.5	38.6	15.1	26.5	35.9	106.8
7	51.5	79.7	125.1	25.8	47.0	73.7	65.8	7.9	12.8	23.4	37.8	11.2	5.1	16.9	39.3	148.4	11.2	36.1
8	44.2	29.0	48.9	22.4	75.8	58.0	59.0	18.3	10.1	30.4	41.5	10.5	19.3	10.8	28.8	46.0	47.9	63.2
9	13.0	8.3	20.1	10.7	14.4	10.4	20.5	3.5	129.4	155.2	3.9	4.9	5.0	7.2	7.0	10.1	9.4	35.4
10	6.5	4.4	7.8	6.1	4.2	5.6	14.6	1.3	32.1	58.3	3.1	1.8	3.1	4.7	4.2	5.2	4.3	8.8

(*b*) Experiment 2

1	54.5	0.4	5.8	3.5	5.0	5.8	62.5	1.8	11.2	13.2	35.3	5.8	11.5	35.3	57.8	26.0	15.1	29.2
2	14.1	0.2	1.2	1.0	2.2	3.6	20.0	0.5	3.4	5.7	3.4	1.6	19.6	8.6	8.5	5.1	187.7	5.5
3	22.4	4.4	10.3	2.5	7.1	15.9	26.2	0.8	41.0	77.2	12.7	7.5	23.0	6.6	6.7	5.3	16.9	22.7
4	48.2	1.4	11.7	5.5	13.8	8.1	34.3	2.3	7.3	10.4	4.9	3.7	2.1	60.0	6.9	5.7	3.8	12.1
5	35.2	1.7	11.7	8.0	9.1	7.2	41.5	0.7	12.3	18.1	1.4	17.6	0.9	20.7	16.1	11.6	1.7	25.6
6	32.3	5.4	7.9	5.0	6.4	6.5	35.9	1.8	32.4	31.9	31.4	19.9	4.5	0.4	4.2	3.5	5.5	27.8
7	11.2	1.1	27.7	11.8	17.2	15.7	16.0	2.7	5.7	7.0	5.9	2.9	1.1	1.5	12.4	47.3	2.0	9.0
8	10.7	3.4	7.8	7.3	16.5	9.7	19.5	4.3	2.5	8.7	5.0	2.4	4.2	0.8	7.6	10.7	8.2	16.8
9	4.1	2.6	4.0	4.2	4.8	1.9	10.6	0.4	37.0	26.6	0.0	1.3	1.5	0.5	2.3	3.1	1.8	7.7
10	2.5	1.0	1.3	3.1	2.7	1.0	7.5	0.2	13.0	13.5	0.0	1.0	1.3	1.5	1.6	1.4	1.2	3.4

(*c*) Sequencing of mock-precipitated material*

1	63.5	5.6	3.6	3.9	8.3	11.3	51.5	2.3	12.2	16.5	8.4	3.5	10.8	47.0	35.2	27.3	12.7	24.4
2	24.8	2.5	3.1	3.6	7.9	6.2	33.8	1.3	6.9	12.1	4.5	1.4	5.8	18.4	7.4	6.4	6.9	13.8
3	15.2	0.9	2.5	3.0	6.6	3.6	26.6	1.2	4.1	11.0	2.7	1.2	4.2	16.1	2.7	4.0	4.3	8.6
4	11.5	1.0	2.2	3.2	5.7	2.6	19.5	0.8	3.9	7.3	2.8	1.1	2.7	10.7	1.6	2.4	3.1	6.4
5	10.5	1.4	2.1	3.1	5.0	2.6	15.7	1.0	3.1	6.2	2.3	0.7	2.2	7.9	0.9	1.7	2.6	5.2
6	8.8	1.1	1.6	3.1	4.1	2.0	12.6	1.1	2.2	4.6	1.9	0.6	1.9	6.5	1.1	1.4	1.9	3.9
7	6.8	1.0	1.6	2.4	3.5	1.8	9.8	0.5	1.8	3.4	2.1	0.4	1.7	4.3	1.6	1.5	1.7	2.7
8	0.0	0.3	0.0	2.1	0.2	0.8	0.8	0.6	1.1	2.8	1.7	0.3	1.1	3.6	0.9	2.2	0.2	2.6
9	0.1	0.6	0.0	1.8	0.0	0.8	0.7	0.2	1.6	2.5	1.7	0.5	1.1	3.3	1.3	1.7	0.1	2.1
10	0.2	0.3	0.0	1.7	0.1	0.5	0.8	0.2	1.0	2.5	1.4	0.3	1.3	2.7	0.8	1.7	0.1	2.1

(*d*) The K^d-restricted peptide motif†

	Position 1	2	3	4	5	6	7	8	9‡
Dominant anchor residues		**Y**							**I L**
Strong			**N I L**	**P**	**M**	**K F**	**T N**		
Weak	K A R S V T	F	A H V R S F E Q K M T	A E S D H N	V N D I L S T G	H I M Y V R L	P H D E Q S	H E K V V F R	

Known epitopes, aligned§										Protein source	Ref.
	T	Y	Q	R	T	R	A	L	V	Influenza PR8 NP 147–154	4, 29
	S	Y	F	P	E	I	T	H	I	Self-peptide of P815	This paper
	I	Y	A	T	V	A	G	S	L	Influenza JAP HA 523-549‖	30, 31
	V	Y	Q	I	L	A	I	Y	A	Influenza JAP HA 523-549‖	30, 31
	I	Y	S	T	V	A	S	S	L	Influenza PR8 HA 518–528	32
	L	Y	Q	N	V	G	T	Y	V	Influenza JAP HA 202–221	30, 31
	R	Y	L	E	N	G	K	E	T	*L*¶ HLA-A24 170-18233	33
	R	Y	L	K	N	G	K	E	T	*L*¶ HLA-Cw3 170-186	34
	K	Y	Q	A	V	T	T	T	L	P815 tumour antigen	35
	S	Y	I	P	S	A	E	K	I	*Plasmodium berghei* CSP 249-260	36
	S	Y	V	P	S	A	E	Q	I	*Plasmodium yoelii* CSP 276-288	37

Fraction 20–28 of K^d-precipitates (*a*, *b*) or mock precipitate (*c*) prepared from $10–20 \times 10^9$ P815 cells according to the precipitates in Fig. 1*a*, *b* were pooled and sequenced by Edman degradation, performed in a pulsed-liquid protein sequencer 477A equipped with an on-line PTH-amino-acid analyser 120A (Applied Biosystems). Glass-fibre filters were coated with 1 mg BioBrene Plus (Applied Biosystems) and were not precycled. Sequencing was carried out using the standard programmes BEGIN-1 and NORMAL-1 (Applied Biosystems). Cys was not modified and therefore not detectable, PTH-Trp coeluted with diphenylurea and, in three out of six experiments, PTH-Arg coeluted with the major derivative of PTH-Thr. Thus, Cys and Trp are not detectable, and Arg only in the absence of Thr. The numbers indicate pmol of individual amino-acid residues detected at each cycle. Numbers indicating more than 50% increase in absolute amount of individual residues compared with their amount in either of the two previous cycles (but not if a value decreases compared with the previous position, or if a value is below 1 pmol) are underlined. These are the residues arbitrarily considered significant at the respective position. At least 10% of a signal is caused by a lag effect from the previous cycle, in addition 10% of Asn is hydrolysed to Asp and 20% of Gln to Glu. Increases in Arg signals can be due to a hydrolysed derivative corresponding to the major derivative of Thr: Thus, increases of Asp in presence of strong Asn signals are not significant; the same is true for Gln–Glu and Arg–Thr pairings, respectively. Material extracted from glycine beads (see Fig. 1) did not indicate particular amino acids except for some glycine residues (not shown).

* The data for material eluted from anti K^b-beads used to mock-precipitate P815 lysates. Sequencing of material from P815 lysates mock-precipitated with anti-D^b-beads showed a very similar pattern, with no increase of any residue compared with its amount at a previous cycle.

† Residues at individual positions were classified into those dominant within anchor positions, or for those with strong or weak signals, according to the magnitude of increase seen for the respective residue.

‡ Anchor positions are indicated in bold types. Classification 'anchor' is only applied if a position reveals a strong signal for only one residue, or alternatively, if a position is occupied by a few residues with closely related side chains.

§ Peptides known to contain K^d-restricted T-cell epitopes were aligned according to their Tyr residues. Amino-acid residues located outside the nonapeptides predicted to be the naturally processed ones are omitted. Peptides known to be the naturally processed ones are underlined. SYFPEITHI is the prominent self-peptide eluting at fraction 29 in Fig. 1*a*, *b* (see text).

‖ This peptide allows two possibilities for alignments with the motif, taking into account the similar side chains of Ile, Leu, Val and Ala.

¶ The residue at position 10 is in italics. These peptides might be exceptional K^d-restricted epitopes having their C-terminal anchor residues at position 10 instead of 9.

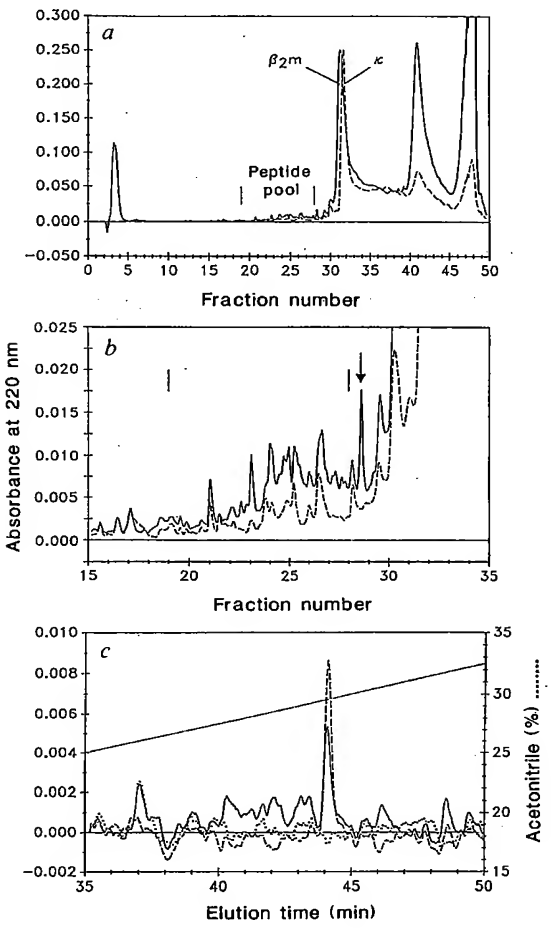

FIG. 1 HPLC separation of immunoprecipitated and TFA-treated K^d molecules. *a*, HPLC profile of TFA-treated material precipitated from P815 lysate with anti-K^d beads (——) or mock-precipitated with irrelevant anti-D^b beads (– – – –). The material eluting beyond fraction 32 is probably MHC light and heavy chains and antibody components. The peak at fraction 48 is detergent. *b*, Enlarged view of the chromatogram in *a* (fraction 15–35). Note several distinct peaks in the K^d precipitate (———), presumably self peptides, which do not appear in the control precipiate (– – – –). The most dominant peak is marked by an arrow (fraction 29) and was determined by sequencing to be SYFPEITHI. *c*, Rechromatography of the dominant self-peptide marked by an arrow in *b*(——), of the corresponding fraction in a control precipitate produced with glycine-coupled beads (· · · ·), and of 500 ng synthetic SYFPEITHI (– – – –).

METHODS. $10–20 \times 10^9$ P815 cells were pelleted and stirred for 30 min with 250 ml 0.5% Nonidet P-40 in phosphate buffered saline (PBS) containing 0.1 mM PMSF at 4 °C. The supernatant (after centrifugation at 250*g*, 5 min, followed by 150,000*g*, 30 min at 4 °C) was passed through a chromatography column (bed volume, 1 ml) filled with glycine-coupled beads, then through a similar column filled with anti-K^d beads, then for a mock-precipitate over anti-D^b beads. Beads removed from all three columns were swirled with 0.1% TFA for 15 min. Supernatants were dried by vacuum centrifugation and separated by reversed-phase HPLC using a Superpac Pep S Column (C2/C18; 5 μm particles, 4.0 × 250 mm; Pharmacia LKB) and Pharmacia LKB equipment[4]. Eluents: solution A, 0.1% TFA in H_2O (v/v); solution B, 0.1% TFA in acetonitrile. Gradient used in *a* and *b*: 0–5 min, 100% A; 5–40 min, linear increase to 60% B; 40–45 min, 60% B, 45–50 min, decrease to 0% B; flow rate 1 ml min^{-1}, fraction size 1 ml. Fraction size in *c*, 0.5 ml. Individual fractions were collected and dried by vacuum centrifugation. Antibody-coupled and glycine-coupled beads were prepared using cyanogen bromide-activated Sepharose 4B (Pharmacia LKB) according to manufacturer's protocol. K^d-specific antibodies (5 mg each) 20-8-4S (IgG2a, κ; ref. 25) or D^b specific B22-249 (IgG2a, κ; ref. 26) were coupled to 1 ml of beads. β2m, β_2-microglobulin.

sequencing. Identity of this natural self-peptide was confirmed by its coelution with synthetic SYFPEITHI on HPLC (Fig. 1*c*). The sequence fits well with the motif from the pool of fractions 20–28 (Fig. 1*a*, *b*), thus confirming the K^d-restricted peptide motif (Table 1*d*). It will be of interest to see whether alloreactive T cells can recognize this prominent self-peptide, which we estimate, on the basis of comparison with the peak area of the coeluting synthetic peptide, to occupy about 5% of K^d molecules of P815 cells (10,000 SYFPEITHI molecules per cell).

Elution of peptides from K^b and D^b

Detergent lysates from EL4 tumour cells ($H\text{-}2^b$) were immunoprecipitated with K^b-specific and D^b-specific antibodies. Peptides dissociated from MHC molecules were separated by reversed-phase HPLC. Both K^b- and D^b-derived material eluted with profiles roughly similar to the K^d-derived material, with marked differences, however, in the heterogeneous material eluting between fractions 20 and 28 (not shown).

D^b-restricted peptide motif

Pooled fractions 20–28 from the D^b preparation were sequenced (Table 2*a*, *b*). Each of positions 2 to 4 contained several residues. By contrast, cycle 5 gave a strong signal for Asn, which was much weaker for cycle 4 (from 4.2 to 271.4 pmol in the first and from 6.7 to 154.7 pmol in the second experiment). Thus, the dominant residue at position 5 of D^b-eluted self-peptides is Asn. The weak signal for Asp is caused by hydrolysis of Asn to Asp under sequencing conditions. Positions 6–8 contained 5–14 different detectable residues. Position 9 contained a strong signal for Met, an intermediate one for Ile, and a weak one for Leu (all hydrophobic). (The importance of Met or Ile in a D^b-restricted epitope has been reported earlier[17].) Position 10 had no signal, indicating D^b-presented self-peptides to be nonapeptides, as is the D^b-restricted influenza peptide[4]. The consensus motif indicated by these data is shown in Table 2*c*. Comparing this motif with the natural D^b-restricted peptide and with other peptides containing D^b-restricted epitopes shows that Asn at position 5 may be an invariant anchor residue (for definition, see Table 1) of the D^b-restricted peptide motif, not unlike Tyr at position 2 for the K^d-restricted motif. The other residues of the D^b-restricted epitopes vary considerably, with the exception of the Met, and also either Ile or Leu at position 9, which looks like a second anchor position. This anchor is not at position 9 in the (few) known T cell epitopes, except in the naturally processed one.

K^b-restricted peptide motif

Pooled fractions 20–28 from the K^b preparation were sequenced (Table 3*a*, *b*). No strong signal for any residue was at the second position. Position 3 contained a good signal for Tyr, and a weak one for Pro. Position 4 revealed weak signals for five residues. Strong signals for Phe (increase from 1.8 to 50.5 and from 1.5 to 18.3 pmol) and for Tyr make these two residues dominant at position 5. The next two positions contained five or three residue signals. Position 8 had a strong signal for Leu, an intermediate one for Met, and weak ones for Ile and Val. Position 9 showed no increase for any residue, consistent with the length of the known K^b-restricted natural peptide, which is an octamer[5]. Analysis of the consensus K^b restricted motif and comparison with epitopes indicates two anchor positions: Tyr or Phe (both with similar aromatic side chains) at position 5 and Leu, Met, Ile or Val (all with similar hydrophobic side chains) at position 8.

HLA-A2.1 restricted peptide motif

Detergent lysate of human JY cells (HLA-A2.1) was immunoprecipitated with A2-specific antibodies. Peptides dissociated from A2 molecules were separated by HPLC. Fractions 20–28 were pooled and sequenced as for the mouse material (Table 4). The second position contained a strong signal for

TABLE 2 Sequencing of the self-peptide mixture eluted from D^b molecules*

(*a*) Experiment 1

Cycle	A Ala	R Arg	N Asn	D Asp	E Glu	Q Gln	G Gly	H His	I Ile	L Leu	K Lys	M Met	F Phe	P Pro	S Ser	T Thr	Y Tyr	V Val
	Amino-acid residues (in pmol)																	
1	257.2	18.2	21.6	1.3	8.1	16.3	99.1	2.3	22.0	21.2	20.3	7.2	33.0	27.5	124.6	43.9	26.9	70.1
2	202.1	7.2	5.4	6.8	7.4	24.7	116.2	0.9	5.4	9.9	6.5	154.1	4.3	8.2	52.7	15.0	5.5	16.0
3	29.9	5.9	5.3	0.0	3.8	5.5	185.1	1.1	106.3	65.8	0.0	8.3	3.8	88.1	8.3	4.7	5.2	73.2
4	18.3	8.1	4.2	4.6	32.4	21.8	49.3	0.8	32.7	21.5	12.4	3.6	2.3	28.8	9.9	18.6	5.0	165.2
5	6.8	2.1	271.4	26.0	8.2	4.3	43.0	0.6	4.7	6.2	2.5	1.3	0.9	11.7	4.5	5.0	1.7	7.6
6	42.1	5.9	29.6	7.1	8.4	7.8	32.6	1.3	18.0	148.4	8.8	1.9	11.3	22.5	7.8	11.8	4.1	23.6
7	21.5	23.4	18.2	24.5	30.4	13.7	22.0	0.7	9.9	16.2	2.4	2.1	3.6	16.4	6.7	54.3	5.1	35.0
8	14.6	10.1	11.3	9.8	23.2	10.3	18.2	0.3	3.0	10.1	4.4	1.3	5.0	9.5	26.5	24.9	12.5	20.7
9	7.5	3.2	7.9	3.2	3.1	1.6	11.2	0.5	8.5	13.7	0.5	7.7	3.0	2.5	2.0	3.3	3.6	3.5
10	2.6	1.1	2.5	2.4	1.9	1.2	12.5	0.3	4.2	8.5	0.4	2.7	1.8	2.1	1.6	1.7	1.9	1.3

(*b*) Experiment 2

1	413.4	45.8	29.7	15.9	14.5	19.6	132.4	4.7	41.5	40.8	48.9	17.2	50.8	26.1	307.7	94.0	47.4	110.1
2	227.4	14.4	7.6	9.3	11.1	25.2	133.8	2.1	8.2	14.5	13.3	169.9	5.6	4.9	71.0	21.6	11.3	22.6
3	39.6	3.3	6.0	6.3	6.0	5.3	172.2	1.2	89.5	56.0	1.6	14.7	4.5	75.4	12.1	5.0	7.6	79.2
4	29.3	16.6	6.7	10.6	34.8	23.0	57.3	0.8	36.3	21.7	17.0	8.1	4.2	33.5	12.5	23.9	7.4	198.9
5	19.9	5.3	154.7	22.2	8.7	4.1	31.1	0.9	4.6	7.0	4.3	2.4	1.7	11.8	5.3	5.0	2.0	13.8
6	42.3	8.4	30.8	15.7	14.6	8.3	28.7	2.3	18.6	124.1	8.2	5.3	11.2	22.1	7.9	10.7	5.6	29.2
7	22.0	24.5	15.4	33.5	29.2	10.5	17.7	1.6	11.3	14.8	3.3	3.7	3.6	14.3	7.5	47.3	6.9	35.5
8	15.8	10.9	10.2	20.9	25.6	8.0	12.6	3.2	3.3	13.6	4.3	2.8	5.1	8.7	20.8	19.3	12.9	23.6
9	8.7	4.3	6.1	13.0	12.1	2.6	8.7	0.3	19.8	26.2	1.2	30.8	3.9	4.4	4.8	5.6	7.2	9.2
10	5.4	3.1	3.9	12.2	8.1	2.0	8.2	0.0	10.1	13.9	0.7	11.6	3.2	3.4	3.0	3.0	7.3	5.9

(*c*) The D^b-restricted peptide motif

	Position 1	2	3	4	5	6	7	8	9†
Dominant anchor residues					**N**				**M**
Strong		**M**	**I** **L** **P** **V**	**K** **E** **Q** **V**		**L** **F**			**I**
Weak	A N I F P S T V	A Q D	G	D T		A Y T V M E Q H I K P S	D E Q V T Y	F H K S Y	L

Known epitopes, aligned‡												Protein source	Ref.
	A	S	N	E	N	M	E	T	M			Influenza NP366-374	4, 2
	S	G	P	S	N	T	P	P	E	/§		Adenovirus E1A	38
	S	G	V	E	N	P	G	G	Y	*C*	*L*§	Lymphocyte choriomeningitis virus GP 272-293	39
	S	A	I	N	N	Y	.	.	.			Simian virus 40 T 193-211‖	40

* Lysates of EL4 cells were prepared as indicated for the P815 lysate in Fig. 1, and then passed through chromatography columns containing glycine beads, anti-K^b (K9-178, IgG2a, κ; ref. 27) or anti-D^b beads. Anti-D^b beads were treated with 0.1% TFA, and the supernatant was separated by HPLC as for the K^d precipitate in Fig. 1. Fractions 20–28 were sequenced and analysed as in Table 1.

† Anchor positions indicated in bold types.

‡ Alignment was on the Asn residue at position 5. The epitope known to be naturally processed is underlined. For the other peptides, only the parts likely to be the naturally processed epitopes are indicated, although most of the peptides were described as longer ones in the original references.

§ Residues at position 10 and 11 are in italics. These peptides might be exceptional D^b-restricted epitopes having their C-terminal anchor residue at position 10 or 11 instead of 9. Note that both epitopes contain four 'small' amino-acid residues, Gly and Pro, which may influence the linear distance between N and C termini.

‖ With the alignment proposed, positions 7–9 are not covered by the peptide published.

Leu and an intermediate one for Met. Positions 3–5 had 6–8 residues each. Position 6 contained Val, Leu, Ile, and Thr. Each of the following two positions had three signals. Position 9 had a strong Val and a weak Leu signal. Position 10 showed no increase for any residue, indicating A2-restricted epitopes to be nonapeptides. Anchors appear to be Leu or Met at position 2 and Val or Leu at position 9. Some of the peptides reported to contain A2-restricted epitopes can be aligned to the motif, whereas others can be aligned only partially (Table 4*c*). The existence of variant A2 molecules typing as A2 by serology may cause the poor alignment to the motif of some of the peptides. The epitope content of some of these peptides has also not been formally established.

Discussion

Sequencing of the self-peptide mixtures from the four MHC class I molecules H-$2K^d$, H-$2K^b$, H-$2D^b$ and HLA-A2 indicated a distinct allele-specific peptide motif presented by each class I molecule. K^d-, D^b- and A2-presented peptides are nonamers, whereas K^b-presented peptides seem to be octamers; the corresponding peptide motifs contain two anchor positions occupied by a fixed residue or by one of a few residues with closely related

TABLE 3 Sequencing of the self-peptide mixture eluted from K^b molecules*

(*a*) Experiment 1

Cycle	A Ala	R Arg	N Asn	D Asp	E Glu	Q Gln	G Gly	H His	I Ile	L Leu	K Lys	M Met	F Phe	P Pro	S Ser	T Thr	Y Tyr	V Val
	Amino-acid residues (in pmol)																	
1	978.7	26.3	49.2	55.8	39.0	23.1	514.9	20.9	167.5	167.2	189.8	50.3	116.7	118.2	120.8	365.2	136.0	352.5
2	345.5	3.9	37.3	41.8	23.5	20.3	475.2	8.9	44.5	43.1	72.6	12.6	25.4	51.0	253.1	80.5	50.1	93.5
3	129.0	1.4	14.7	37.0	17.7	9.8	358.8	5.9	8.2	19.0	26.9	4.1	6.0	32.5	56.2	20.0	75.6	25.9
4	52.1	3.5	10.8	45.3	38.0	9.2	246.7	5.0	4.9	7.0	17.7	2.4	1.8	14.6	23.0	13.4	12.0	16.4
5	18.9	1.3	5.5	34.7	12.0	3.6	128.2	2.8	1.9	4.7	3.8	1.6	50.5	6.7	8.9	4.6	33.2	4.9
6	16.2	0.8	5.6	32.7	13.0	3.7	77.9	2.4	3.1	3.5	3.9	0.9	4.5	7.3	9.2	18.3	7.3	6.2
7	9.9	0.9	14.9	30.4	9.5	6.6	51.3	0.6	0.0	3.4	9.2	0.5	1.9	4.7	6.1	10.7	3.5	3.4
8	6.0	1.4	5.1	22.7	6.0	3.3	29.2	0.8	1.4	13.5	1.8	2.1	1.0	3.8	4.1	3.1	2.5	3.6
9	4.6	1.5	2.6	19.9	4.5	2.3	21.1	0.9	0.9	6.9	1.0	1.5	1.0	3.0	3.7	2.2	1.9	2.1
10	3.9	0.5	1.9	17.5	3.7	2.1	17.5	1.0	0.5	4.0	0.8	0.9	1.2	2.8	3.5	1.8	2.0	1.5

(*b*) Experiment 2

1	42.4	1.1	5.2	3.0	7.8	17.1	44.6	0.3	11.3	12.6	12.1	3.8	6.2	7.6	44.2	18.1	6.8	26.2
2	24.0	0.2	9.4	2.8	5.1	8.0	42.5	0.5	4.7	6.3	4.0	1.3	3.7	3.5	14.9	10.3	3.1	6.9
3	10.4	0.3	2.1	2.6	3.9	4.0	25.1	0.7	2.8	7.9	2.1	0.9	3.6	9.8	3.0	3.3	16.7	10.0
4	9.6	1.3	2.7	5.7	7.5	4.1	24.5	0.2	1.5	5.0	6.3	0.7	1.5	5.9	3.0	5.9	2.7	4.5
5	5.8	0.8	1.8	2.8	3.3	2.5	14.2	0.5	0.2	3.9	1.7	0.4	18.3	3.5	1.3	2.0	20.8	2.2
6	8.6	0.2	2.3	2.7	6.3	2.7	9.2	0.0	1.0	2.4	1.5	0.4	2.3	3.2	2.7	5.2	3.6	2.4
7	5.0	0.1	8.2	3.3	3.9	4.2	10.4	0.6	0.4	2.3	7.2	0.1	1.2	2.1	1.9	2.8	1.9	1.2
8	4.0	0.1	3.1	2.0	2.6	1.7	6.9	0.2	0.2	13.8	1.6	1.0	0.8	1.1	0.7	1.3	1.1	2.2
9	4.5	0.1	1.1	2.1	3.6	1.9	5.9	1.4	0.0	7.7	0.9	1.0	0.9	1.3	0.3	1.3	0.8	1.7
10	3.9	1.7	0.3	4.5	3.0	1.4	5.4	0.2	0.0	3.9	0.6	0.6	0.6	1.1	0.6	1.1	0.8	1.1

(*c*) The K^b-restricted peptide motif

	Position 1	2	3	4	5	6	7	8†
Dominant anchor residues					**F** **Y**			**L**
Strong			**Y**					**M**
Weak	R I L S A	N	P	R D E K T		T I E S	N Q K	I V

Known epitopes, aligned‡									Protein source	Ref.
	R	G	Y	V	Y	Q	G	L	Vesicular stomatitis virus NP 52-59	5
	S	I	I	N	F	E	K	L	Ovalbumin 258–276§	41
	A	P	G	N	Y	P	A	L	Sendai Virus NP 321-332	42

* The anti-K^b beads loaded with EL4 lysates described in Table 2 were treated with 0.1% TFA as were the anti-K^d beads in Fig. 1. Extracted material was HPLC-separated as before, and fractions 20–28 were pooled, sequenced, and analysed as in Table 1.

† Anchor positions are in bold types.

‡ Alignment was on the Leu residue at position 8. The only known naturally processed epitope is underlined. For the others, only the postulated natural epitopes are indicated; additional residues contained in the original references have been omitted.

§ The published ovalbumin peptide did not contain the Ser residue at position 1.

side chains. These anchor positions are not at the same place in the different motifs; they are at position 5 and 9 (D^b), or 2 and 9 (K^d, A2), or 5 and 8 (K^b). The C-terminal anchor residues of all motifs are hydrophobic. For H-$2L^d$ (data not shown), one anchor residue was Pro at position 2. (The entire motif could not be sequenced, as not enough material eluted from L^d molecules.) The residues not at anchor positions can be fairly variable; some, however, seem to be preferentially occupied; for example, Pro is prominent at position 4 of the K^d motif, Tyr at position 3 of the K^b motif, and hydrophobic residues predominate at positions 3 of the D^b motif and 6 of the A2 motif. Because of technical limitations of our unconventional approach, less abundant amino-acid residues may have escaped detection; Cys and Trp residues were not detected at all. Comparison of the motifs with known epitopes indicates that some residues have been missed by sequencing the self-peptide mixtures. A minor population of peptides not adhering to the motif-specific lengths also cannot be excluded. The K^d- and D^b-restricted epitopes whose positions 9 do not fit with the hydrophobic anchor of the respective motifs would have residues at position 10 or 11 that would fit (Tables 1*d*, 2*c*).

Our observations match well the structure of the peptide-binding cleft of MHC class I molecules[3,6]. With HLA-A2, the cleft has pockets of a size that would accommodate specific amino-acid side chains, for example Leu[3,6]. Leu dominantly occupies position 2 of the A2 motif. Another pocket should exist in A2 molecules to accommodate the side chains of Val and Leu at position 9 of A2-restricted epitopes. Co-crystallizing material not from the A2 sequence and bound to the cleft showed extensions (possibly Leu and Val side chains) fitting the A2 pockets[3,6]. With the Aw68 crystal, which differs from A2 at 13 amino-acid residues, pockets of different location and of different shape from that of A2 were found[6]. Therefore, different MHC class I alleles differ in the location and shape of pockets in the cleft likely to be able specifically to accommodate certain amino-acid side chains.

Thus, the allele-specific pockets in MHC crystals and the side chains of the allele-specific anchor residues we find in the sequenced self-peptides are likely to reflect complementary structures. From this, we deduce that the structure of K^d (mouse MHC molecules have not been crystallized yet) possesses a pocket for the aromatic side chains of Tyr or Phe at one end of the cleft and another one at the opposite end, able to accommodate Ile, Leu, or Val (all hydrophobic with side-chain methyl groups). Similarly, K^b should have a pocket close to the cleft's centre for the aromatic side chains of Tyr or Phe, and another

TABLE 4 Sequencing of the self-peptide mixture eluted from A2.1 molecules*

(*a*) Experiment 1

Cycle	A Ala	R Arg	N Asn	D Asp	E Glu	Q Gln	G Gly	H His	I Ile	L Leu	K Lys	M Met	F Phe	P Pro	S Ser	T Thr	Y Tyr	V Val
	Amino-acid residues (pmol)																	
1	172.6	0.0	31.9	25.7	44.8	125.9	112.4	2.8	144.4	123.8	60.0	30.7	63.3	117.9	75.9	49.0	50.3	104.9
2	42.5	0.0	16.2	14.1	25.6	53.1	44.7	1.6	69.6	511.0	15.5	71.0	10.5	38.7	16.2	16.1	12.2	86.5
3	99.8	0.0	9.5	18.3	12.3	20.4	31.8	11.1	51.5	110.8	5.8	55.7	19.4	30.4	12.0	8.7	20.9	46.0
4	36.0	0.6	12.7	26.4	59.5	21.7	56.2	1.3	10.4	22.7	24.6	5.2	5.2	52.4	10.9	14.0	5.2	28.8
5	35.1	0.1	13.4	18.6	28.1	19.8	55.6	2.8	21.4	23.9	47.2	4.1	6.2	39.1	7.5	10.5	11.6	29.0
6	30.3	0.9	16.8	14.1	21.4	17.3	28.5	1.4	68.1	43.4	14.7	4.4	5.8	40.8	9.2	20.3	5.0	106.2
7	42.1	0.0	11.7	9.5	27.2	21.8	19.0	3.2	36.3	27.3	7.9	5.7	8.0	54.1	5.4	13.6	14.0	62.8
8	37.9	0.3	13.4	8.1	37.3	24.3	21.1	1.8	11.6	15.1	33.8	3.4	5.1	22.3	8.8	17.9	10.2	22.4
9	23.3	0.0	5.1	6.0	15.7	10.5	14.8	0.7	11.5	27.5	8.7	3.1	2.7	11.9	5.6	6.7	5.1	60.2
10	12.0	0.7	2.6	4.4	6.5	5.2	10.2	0.4	4.5	12.1	4.5	1.0	1.8	7.1	2.7	3.2	2.3	20.4

(*b*) Experiment 2

1	110.8	10.8	4.0	3.1	10.0	14.5	55.7	0.2	60.3	44.4	10.8	8.2	37.5	20.3	27.4	14.6	19.8	48.0
2	13.4	1.6	2.0	1.9	6.8	11.0	9.0	0.0	37.9	302.7	0.0	26.2	5.0	6.3	4.4	4.5	3.3	26.5
3	62.4	3.5	5.0	9.1	4.9	10.0	12.6	0.1	35.7	71.5	0.0	24.5	13.8	13.4	8.9	4.8	17.9	19.6
4	16.9	2.2	4.5	8.9	25.3	7.9	24.5	0.1	6.2	10.3	2.8	1.3	2.0	22.1	4.9	5.0	1.8	9.3
5	22.3	1.6	6.8	8.6	14.3	9.9	31.8	0.0	16.6	15.1	8.2	1.9	4.0	16.3	4.5	4.6	5.7	18.3
6	10.6	1.3	6.6	3.6	6.4	6.2	10.1	0.1	38.7	27.1	0.0	1.4	2.7	12.6	3.2	6.1	1.3	39.2
7	19.2	1.0	4.7	2.5	7.2	9.0	5.6	0.2	22.3	16.1	0.0	1.9	3.9	17.4	1.9	3.5	3.6	27.2
8	13.4	1.2	3.1	1.3	7.9	6.3	6.9	0.3	4.7	6.7	3.0	0.6	2.0	5.1	2.2	4.9	1.6	5.3
9	5.7	0.5	0.9	0.8	2.9	2.0	2.7	0.2	3.8	11.5	0.4	0.3	0.6	2.0	1.0	1.1	0.4	10.8
10	2.9	0.6	0.5	0.5	1.0	0.9	1.8	0.3	1.6	5.1	0.4	0.3	0.3	0.8	0.4	0.3	0.2	3.6

(*c*) The HLA-A2-restricted peptide motif

	Position 1	2	3	4	5	6	7	8	9†
Dominant anchor residue		**L**							**V**
Strong		**M**		**E**		**V**		**K**	
				K					
Weak	I		A	G	I	I	A	E	L
	L		Y	P	K	L	Y	S	
	F		F	D	Y	T	H		
	K		P	T	N				
	M		M		G				
	Y		S		F				
	V		R		V				
					H				

Reported epitopes, aligned‡											Protein source	Ref.
	I	L	K	E	P	V	H	G	V		HIV reverse transcriptase 461–485	43
	G	I	L	G	F	V	F	T	L		Influenza matrix protein 57–68	44
	I	L	G	F	V	F	T	L	T	*V*¶	Influenza matrix protein 57–68	44
	F	L	Q	S	R	P	E	P	T		HIV Gag protein 446–460§	46
	A	M	Q	M	L	K	E	.	.		HIV Gag protein 193–203§	46
	P	I	A	P	G	Q	M	R	E		HIV Gag protein 219–233§	46
	Q	M	K	D	C	T	E	R	Q		HIV Gag protein 418–443§	46

* Detergent lysate of JY cells (HLA-A2.1)[45] prepared as indicated for P815 cells in Fig. 1 was passed through a column containing glycin beads, then through a column containing anti-A2 (BB7.2, IgG2b; ref. 28) beads. Loaded anti-A2 beads were treated with 0.1% TFA, and the material in the supernatant was separated by HPLC as for the K^d preparation in Fig. 1. Fractions 20 to 28 were pooled and sequenced as in Table 1.

† Anchor positions are in bold types.

‡ Alignment was on the anchor at position 2 of the motif. Only the nonapeptides fitting to the motif are indicated, although all of the peptides were described as longer ones in the original references. For the influenza matrix peptide, an alternative alignment is shown, which employs an Ile residue at position 2. Ile and Leu have very similar side chains. With this alignment, position 9 of the peptide fits with the C-terminal anchor of the motif. In addition, position 6 of the peptide then fits well with position 6 of the motif, which may represent an auxiliary anchor of this motif preferentially for hydrophobic residues.

§ All four HIV Gag peptides can only partially be aligned with the motif. These peptides induced low or intermediate lysis of target cells at micromolar concentrations[46], whereas natural epitopes induce lysis at picomolar concentrations[4]. All four peptides were recognized by the same cytotoxic T lymphocyte line[46].

¶ The residue at position 10 is in italics. This peptide might be an exceptional A-2 restricted epitope having its C-terminal anchor residue at position 10 instead of 9.

at the very end of the cleft for Leu or Met, which have hydrophobic side chains similar to each other. For D^b we postulate a central pocket exclusively for Asn. A second pocket for Met, Ile, Leu, or Val should be at one end of the D^b-cleft. This pocket might be less specific, as some peptides reported to contain D^b-restricted epitopes do not fit to the motif at this position. Alternatively, these peptides may represent examples of a minority of epitopes having their C-terminal hydrophobic anchor residue at position 10 or 11 (Table 2*c*). L^d molecules should contain a pocket for Pro at one end of the groove.

The mechanism by which MHC-restricted epitopes are produced is unclear, even when they stem from known proteins. As all epitopes appear to have hydrophobic C termini, whereas the N termini are highly variable, the hydrophobic end might be a site for an MHC-independent protease[8], producing larger precursor peptides, whose N termini are trimmed after binding to MHC[8].

Strictly allele-specific peptide motifs seem to be contradicted by data obtained from assays measuring MHC binding to solid-phase peptides (for example, refs 19, 20); later studies however suggest that this reflects the artificial situation in which these assays are done. We have also shown that artificial peptides (not adhering to MHC restricted motifs) binding to MHC molecules, and even inducing T-cell responses are not processed

and presented by cells expressing the respective protein[21]. From this we conclude that more peptides can bind to MHC molecules than are processed by cells. Taking into account the strong adherence of naturally processed peptides to the MHC-restricted motifs, we also conclude that these motifs are not 'peptide binding motifs', but motifs representing the outcome of processing, which is likely to include MHC-dependent and MHC-independent protease activities[8,9] and transport mechanisms[22–24]. Intracellular binding to MHC molecules of epitopes correctly processed without participation of MHC molecules cannot entirely be excluded, although it is unlikely, given our inability to find intracellularly correctly processed epitopes independent of MHC[8]. Thus peptide binding to MHC molecules is a necessary requirement for a peptide being an MHC-restricted epitope but is not sufficient on its own.

Knowledge of the peptide motifs of individual MHC alleles should help exact T-cell epitope predictions (K.F. *et al.*, manuscript submitted; K. Deres *et al.*, unpublished) and help with synthetic or recombinant vaccine development and potentially also for intervention in autoimmune diseases or graft rejection. □

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Statistical evidence for a galactic origin of gamma-ray bursts

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ASTRONOMICAL bursts of gamma-rays (GRBs) were first discovered 20 years ago, and ~100 are recorded every year by satellite-borne instruments. Bursts last for at most a few seconds, recur only on a timescale of years, if at all, and come from objects which have remained undetected at all other wavelengths. It has been impossible to establish a distance scale for GRBs, and no association with known astronomical objects has been demonstrated. Here we analyse the spatial distribution of GRBs[1,2] with a view to understanding their true radial distribution. Our data consist of three samples, totalling 244 GRBs, obtained by three French–Soviet experiments flown on the Venera 13 and 14 and the Phobos missions. We conclude that the underlying GRB distribution is not uniformly distributed in space, but falls off with distance. Our analysis of the weak sources in particular suggests that GRBs are associated with the galactic plane.

There has recently been interest in using the V/V_{max} distribution to study the radial distribution of the sources[3,4], where for a given burst V is the volume of the smallest sphere containing the source, and V_{max} is the maximum volume accessible to the instrument. This ratio is defined as follows: if D is the (unknown) distance of the GRB source and D_{max} the maximum distance at which it can be detected, then $V/V_{max} = (D/D_{max})^3$, and as the number of detected counts, C, scales as D^{-2}, we can write $V/V_{max} = (C_{max}/C_{min})^{-3/2}$ where C_{max} is the maximum number of counts and C_{min} the minimum number required to detect a burst. The V/V_{max} distribution is free of instrumental selection effects as long as C_{max} and C_{min} are calculated in the same time intervals and energy range as those used to trigger the experiment[3] (detailed analysis shows that some bias can still affect the V/V_{max} distribution; Hartmann *et al.*, manuscript in preparation). If the number of sources per unit volume is constant, corresponding to an infinite uniform density population, the V/V_{max} values are uniformly distributed between 0 and 1. Here we present the V/V_{max} distribution for bursts detected by the Signe experiments on the Soviet Venera 13 and 14 probes, and by the Lilas and Apex experiments on the Phobos probe. We use both the mean value $\langle V/V_{max} \rangle$ and a Kolmogorov–Smirnov (KS) test to compare the observed V/V_{max} distribution with a uniform one. Two of the three data sets exhibit a significant deviation from a uniform distribution, which may be interpreted as a deficit of sources at large distances. We show that the faintest sources (in the V/V_{max} sense) seem to be concentrated in the galactic plane, evidence which supports a galactic-disk distribution of GRB sources. The V/V_{max} analysis has been used previously to study the radial distribution of burst samples[4–7], but here we associate a low $\langle V/V_{max} \rangle$ value with the observation of angular anisotropy.

Table 1 gives the main characteristics of the three experiments used here (details available for Signe[8] and in preparation for Lilas and Apex (C. Barat *et al.*, manuscript in preparation)). The Signe experiment had four identical detectors, the Lilas

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Identification of an Enhancer Agonist Cytotoxic T Lymphocyte Peptide from Human Carcinoembryonic Antigen

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ABSTRACT

A vaccination strategy designed to enhance the immunogenicity of self-antigens that are overexpressed in tumor cells is to identify and slightly modify immunodominant epitopes that elicit T-cell responses. The resultant T cells, however, must maintain their ability to recognize the native configuration of the peptide-MHC interaction on the tumor cell target. We used a strategy to enhance the immunogenicity of a human CTL epitope directed against a human self-antigen, which involved the modification of individual amino acid residues predicted to interact with the T-cell receptor; this strategy, moreover, required no prior knowledge of these actual specific interactions. Single amino acid substitutions were introduced to the CAP1 peptide (YLSGANLNL), an immunogenic HLA-A2$^+$-binding peptide derived from human carcinoembryonic antigen (CEA). In this study, four amino acid residues that were predicted to potentially interact with the T-cell receptor of CAP1-specific CTLs were systematically replaced. Analogues were tested for binding to HLA-A2 and for recognition by an established CTL line directed against CAP1. This line was obtained from peripheral blood mononuclear cells from an HLA-A2$^+$ individual vaccinated with a vaccinia-CEA recombinant. An analogue peptide was identified that was capable of sensitizing CAP1-specific CTLs 10^2–10^3 times more efficiently than the native CAP1 peptide. This enhanced recognition was shown not to be due to better binding to HLA-A2. Therefore, the analogue CAP1-6D (YLSGADLNL, Asn at position 6 replaced by Asp) meets the criteria of a CTL enhancer agonist peptide. Both the CAP1-6D and the native CAP1 peptide were compared for the ability to generate specific CTL lines *in vitro* from unimmunized apparently healthy HLA-A2$^+$ donors. Whereas CAP1 failed to generate CTLs from normal peripheral blood mononuclear cells, the agonist peptide was able to generate CD8$^+$ CTL lines that recognized both the agonist and the native CAP1 sequence. Most importantly, these CTLs were capable of lysing human tumor cells endogenously expressing CEA. The use of enhancer agonist CTL peptides may thus represent a new efficient direction for immunotherapy protocols.

INTRODUCTION

A major challenge of modern cancer immunotherapy is the identification of CTL epitopes from defined TAAs[3] that promote lysis of tumor cells. The majority of antigens in human cancers, rather than being tumor specific, are overexpressed in malignant cells as compared to normal tissues. Immunity to cancer in humans may be mainly directed to self-molecules, thus posing the challenge of developing an efficient immune response.

Human CEA is a 180-kDa glycoprotein expressed in the majority of colon, rectal, stomach, and pancreatic tumors (1), 50% of breast carcinomas (2), and 70% of lung carcinomas (3). CEA is also expressed in fetal gut tissue and, to a lesser extent, in normal colonic epithelium. The immunogenicity of CEA has been controversial; several studies reported the presence of antibodies to CEA in patients (4–7), whereas other investigations were unsuccessful (8–10). We have recently reported the first evidence for a human CTL response to CEA (11). We identified a 9-mer peptide designated CAP1 (YLSGANLNL) on the basis of binding to HLA-A2 and the ability to generate specific CTLs from PBMCs from carcinoma patients immunized with rV-CEA. Two other laboratories have since generated CAP1-specific CTLs *in vitro* using peptide-pulsed dendritic cells as antigen-presenting cells (12).[4] It has also recently been reported (13) that CAP1-specific CTLs can be generated from PBMCs from carcinoma patients immunized with the avipox recombinant ALVAC-CEA. Several groups have also reported the generation of anti-CEA antibodies and CEA-specific proliferative T-cell responses after immunization with either an anti-idiotype to an anti-CEA mAb (14), recombinant CEA protein (15), or rV-CEA (16).

Several investigators have induced CTLs to TAAs and viral antigens by *in vitro* stimulation of PBMCs with an immunodominant peptide. Recent work with the gp100 melanoma antigen (17–19), an HIV polymerase peptide (20), and the papilloma virus tumor antigen E6 (21) demonstrated enhanced immunogenicity after modifications to the peptide sequences. In these studies, replacements were at anchor positions and were intended to increase binding to murine or human MHC antigens. This approach was based on the demonstrated correlation between immunogenicity and peptide binding affinity to class I MHC molecules for viral antigen epitopes (22).

In the case of CAP1, the primary and secondary anchors at positions 2, 9, and 1 are already occupied by preferred amino acids. Therefore, we took a different approach to improve peptide immunogenicity by attempting to enhance its ability to bind to the TCR. We proposed that by altering amino acid residues expected to contact the TCR, one could generate a T-cell enhancer agonist, *i.e.*, an analogue with substitutions at non-MHC anchor positions that stimulates CTLs more efficiently than the native peptide. In this we were encouraged by several previous reports supporting the notion that some peptide analogues may act as T-cell antagonists by inhibiting responses to the antigenic peptide (23–29). In these cases, the inhibition was shown to be TCR specific and could not be explained by competition for peptide binding to the MHC protein. By analogy, a peptide enhancer agonist would be an analogue that increased effector function without accompanying increases in MHC binding. In the present report, we attempted to increase CAP1 immunogenicity by analyzing a panel of 80 analogues containing single amino acid substitutions to residues predicted to interact with the TCR of CAP1-specific CTLs. We describe here the construction of such a T-cell enhancer agonist for the CAP1 peptide; to our knowledge, this is the first such example for a human CTL epitope.

MATERIALS AND METHODS

Peptides. A panel of single amino acid substitutions to positions p5–p8 of the CEA peptide CAP1 were made by f-moc chemistry using pin technology (Chiron Mimotopes, Victoria, Australia). CAP1 (YLSGANLNL) and

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[3] The abbreviations used are: TAA, tumor-associated antigens; CEA, carcinoembryonic antigen; TCR, T-cell receptor; PBMC, peripheral blood mononuclear cells; FBS, fetal bovine serum; IL, interleukin; ATCC, American Type Culture Collection; rV-CEA, recombinant vaccinia virus expressing CEA; MFI, mean fluorescence intensity; mAb, monoclonal antibody.

[4] S. Nair and E. Gilboa, personal communication.

CAP1-6D (YLSGADLNL), greater than 96% pure, were also made by Multiple Peptide Systems (San Diego, CA). Additional peptides CAP1-7I and NCA571 were synthesized on an Applied Biosystems 432A synthesizer and were greater than 90% pure by C18 reverse-phase high-performance liquid chromatography.

Cell Lines. T-Vac8[5] and T-Vac24 (11) are human CTLs specific for the CEA peptide CAP1. These cell lines were generated by *in vitro* stimulation of PBMCs using CAP1 and IL-2 according to previously published methods (11). Briefly, postimmunization PBMCs were from HLA-A2$^+$ individuals with advanced carcinoma that had been given rV-CEA in a Phase I trial. PBMCs were isolated on gradients of lymphocyte separation medium (Organon Teknika, Durham, NC), and 2×10^5 cells were placed in wells of sterile 96-well culture plates (Corning Costar, Cambridge, MA) along with 50 μg/ml peptide. After 5 days of incubation at 37°C in a humidified atmosphere containing 5% CO_2, supernatants were removed and replaced with medium containing 10 units/ml human IL-2 (a gift of the Surgery Branch, National Cancer Institute). Cultures were fed with IL-2 every 3 days for 11 days and then restimulated with irradiated (4000 rad) autologous PBMCs (5×10^5) and peptide. Fresh IL-2 was provided every third day, and subsequent restimulations were done every 2 weeks. CTLs were maintained in complete RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with glutamine (Life Technologies, Inc.), penicillin, streptomycin, and 10% pooled human AB serum (Gemini Bioproducts, Inc., Calabasas, CA).

Cell line C1R-A2 (provided by Dr. W. Biddison, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD) was maintained in complete RPMI 1640 with 10% FBS (Biofluids, Rockville, MD), glutamine, nonessential amino acids and pyruvate (Biofluids), and 1 mg/ml G418. Cell line 174.CEM-T2 (provided by Dr. P. Creswell, Yale University School of Medicine, New Haven, CT) is defective in endogenous peptide processing and was maintained in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) with 10% FBS. Both C1R-A2 and T2 lines present exogenous peptides with HLA-A2.

CEA-positive tumor cell lines SW480, SW1463, SW1116, and SW837 were obtained from ATCC (Rockville, MD) and passaged weekly in the respective culture medium described in the ATCC catalogue. The CEA-negative melanoma line SKmel24 (provided by Dr. S. Rosenberg, National Cancer Institute) was passaged weekly in RPMI 1640, 10% FBS, and 10 μg/ml gentamicin (Life Technologies, Inc.). The CEA-negative ovarian tumor cell line CaOV3 was provided by Dr. R. Freedman (M. D. Anderson Cancer Center, Houston, TX) and cultured in RPMI 1640 with 15% FBS, glutamine, 12 μg/ml insulin (Sigma, St. Louis, MO), 10 μg/ml hydrocortisone (Biofluids), and 10 μg/ml gentamicin. All tumor lines were trypsinized with trypsin/versene (Biofluids) for 5–10 min before labeling with isotope for CTL assays. The highly sensitive natural killer target K562 was obtained from ATCC and passaged weekly with RPMI 1640 and 10% FBS.

Generation of CTLs. T-cell lines T-N1 and T-N2 were generated from PBMCs of two normal HLA-A2-positive donors by *in vitro* stimulation with peptide as follows. For the first stimulation cycle, T cells were positively selected by panning on CD3$^+$ MiroCellector flasks (Applied Immune Sciences, Santa Clara, CA). CD3$^+$ cells (3×10^6) were cultured with 10^6 174.CEM-T2 cells that were previously infected with vaccinia virus expressing human B7[6] at a multiplicity of infection of 10, pulsed with 50 μg/ml CAP1 or CAP1-6D peptide and 2 μg/ml human β2 microglobulin (Intergen, Purchase, NY), and irradiated (10,000 rad). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in T25 flasks in RPMI 1640 with 10% human serum, 2 mM glutamine, and 10 μg/ml gentamicin in a total volume of 10 ml with 2×10^7 irradiated (2,500 rads) autologous PBMCs as feeder cells. After 24 h in culture, 10 units/ml human IL-2 and 0.1 ng/ml recombinant IL-12 (R&D Systems, Minneapolis, MN) were added. After 9 days in culture, cells were restimulated using irradiated (10,000 rads) autologous EBV-B cells preincubated with 25 μg/ml peptide at a ratio of 2.5:1 stimulator cells to T cells, and IL-2 and IL-12 were again added 24 h later. Peptide concentration was halved with each subsequent stimulation cycle until a final concentration of 3.12 μg/ml was achieved. CTL experiments were performed using bulk cultures (not clones).

In addition, CTLs were generated from postimmunization PBMCs of cancer patient Vac8 by stimulation with CAP1-6D according to already published procedures (11).

CTL Assay. Target cells were labeled with ^{51}Cr or ^{111}In and then incubated at 2,000–10,000 cells/well with or without peptides in round-bottom microtiter plates (Corning Costar). One h later, T cells were added. Supernatants were harvested (Skatron, Inc., Sterling VA) after 4 h, and isotope release was measured. All assays were performed in triplicate, and the percentage of specific release was calculated as: (observed release − spontaneous release)/(maximum release − spontaneous release) × 100, in which spontaneous release is obtained by omitting the T cells, and maximum release is obtained by adding 1% Triton X-100.

Binding Assay. Binding of peptides to HLA-A2 was evaluated by incubation with processing-defective 174.CEM-T2 cells and measuring the stability of cell surface peptide-A2 complexes (30). Briefly, cells were harvested and washed with serum-free RPMI 1640 and then incubated overnight at $1–2 \times 10^6$ cells/well with various concentrations of peptides. The next day, cells were collected; washed in PBS with Ca^{2+}, Mg^{2+}, and 5% FBS; and then divided into aliquots for single-color flow cytometric analysis. Cells were incubated for 1 h on ice without antibody, with anti-A2 antibody A2.69 (One Lambda, Inc., Canoga Park, CA), or with isotype-matched control antibody UPC-10 (Organon Teknika) and then washed and stained for 1 h with FITC goat antimouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL). Cell surface staining was measured in a Becton Dickinson flow cytometer (Mountain View, CA), and MFI for 10,000 live cells was plotted against peptide concentration. Dissociation experiments were performed as described previously (20). Briefly, T2 cells were incubated overnight with β2 microglobulin and 100 μg/ml peptide and then washed free of unbound peptide and incubated with brefeldin A (Sigma) to block delivery of new class I molecules to the cell surface. At the indicated time points, aliquots of cells were removed, and cell surface peptide-HLA-A2 complexes were immediately quantified as described above.

TCR Chain Usage. T-N1 CTLs were cultured as described for five cycles of antigenic stimulation using the CAP1-6D analogue. The line was then split, and duplicate cultures were maintained with either CAP1 or CAP1-6D for five additional stimulation cycles. Ficoll-purified T cells (5×10^5) were stained with a panel of 19 anti-Vβ and 2 anti-Vα murine mAbs to human $\alpha\beta$ TCR variable regions. Cells were incubated with 10 μg/ml purified antibodies for 30 min at 4°C. The unlabeled mAbs used were Vβ3.1 clone 8F10, Vβ5(a) clone 1C1, Vβ5(b) clone W112, Vβ5(c) clone LC4, Vβ6.7 clone OT145, Vβ8(a) clone 16G8, Vβ12 clone S511, Vβ13 clone BAM13, Vα2 clone F1, and Vα12.1 clone 6D6 (T Cell Diagnostics, Woburn, MA) and Vβ18 (Immunotech, Westbrook, ME). Cells were stained with 10 μg/ml FITC-labeled goat antimouse IgG antibody (Southern Biotechnology Associates) for 30 min in the dark. Directly labeled mAbs were FITC-labeled Vβ11, Vβ21.3, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ20, and Vβ22 and phycoerythrin-labeled Vβ9 and Vβ23 (Immunotech). Cells were fixed with 1% paraformaldehyde, washed with FACSFlow buffer (Becton Dickinson), and analyzed using a Becton Dickinson flow cytometer.

RESULTS

Effect of CAP1 Amino Acid Substitutions on CTL Activity. Several factors were considered in deciding which positions to examine for effects on T-cell activity. Sequencing and mapping experiments have defined a binding motif in which position 2 and the carboxyl-terminal (position 9 or 10) are critical for peptide presentation by HLA-A2 (for review, see Ref. 31). In addition, Tyr at position 1 has been identified as an effective secondary anchor (20, 32). Because the CEA peptide CAP1 already has the preferred amino acids at these three positions, these residues were not altered. Instead, we focused attention on residues predicted to interact with the TCR in the hope of finding analogues that would stimulate human CAP1-specific cytotoxic T cells. X-ray crystallographic studies of several peptides bound to soluble HLA-A2 suggest that all binding peptides assume a

[5] K. Y. Tsang, M. Z. Zhu, C. A. Nieroda, P. Correale, S. Zaremba, J. M. Hamilton, D. Cole, C. Lam, and J. Schlom, Phenotypic stability of a cytotoxic T cell line directed against an immunodominant epitope of human carcinoembryonic origin.

[6] E. Bergmann, J. Schlom, and S. Abrams, manuscript in preparation.

common conformation in the peptide binding groove (33). When five model peptides were examined, residues 5–8 protrude away from the binding groove and are potentially available for binding to a TCR. Therefore, a panel of 80 CAP1 analogue peptides was produced in which the residues at positions 5–8 (p5–p8) were synthesized with each of the 20 natural amino acids. The peptides are designated CAP1-pAA, where p refers to the position in the peptide, and AA refers to the replacement amino acid, using the single-letter amino acid code; *i.e.*, CAP1-6D in which position 6 is occupied by aspartic acid.

The effects of these amino acid substitutions on potential TCR recognition were studied using a CAP1-specific HLA-A2-restricted human CTL line designated T-Vac8. Briefly, T-Vac8 was generated as described in "Materials and Methods" by *in vitro* peptide stimulation of PBMCs from a patient that had been given rV-CEA. For initial screening, T-Vac8 was used in a cytotoxicity assay to measure ^{111}In release from labeled C1R-A2 cells incubated with each member of the peptide panel (at three peptide concentrations). Spontaneous release from the targets (in the absence of T-Vac8) was determined for each individual peptide.

The results are presented in Fig. 1. Of the 80 single amino acid substitutions, most failed to activate cytotoxicity of T-Vac8. However, six independent substitutions preserved reactivity. At position 5, analogues CAP1-5F, CAP1-5I, and CAP1-5S provided stimulation, albeit at reduced levels compared to CAP1 itself. At position 6, the substitutions CAP1-6C and CAP1-6D activated T-Vac8 cytotoxicity and seemed to be equal to or better than CAP1, because they were more active at the intermediate (0.1 μg/ml) peptide concentration. At position 7, analogue CAP1-7I also seemed to be active. Finally, at position 8, no analogues were able to sensitize targets to lysis by T-Vac8./The two most active analogues (CAP1-6D and CAP1-7I) were then analyzed in detail, omitting CAP1-6C due to concern for disulfide formation under oxidizing conditions.

Purer preparations (90–96% pure) of native CAP1 and the analogues CAP1-6D and CAP1-7I were synthesized and compared in a CTL assay over a wider range of peptide concentrations using two different cell lines as targets (Fig. 2). Using T2 cells, analogue CAP1-6D was at least 10^2 times more effective than native CAP1. CAP1-6D lytic activity was half-maximal at 10^{-4} μg/ml (Fig. 2*A*). In contrast, the CAP1-7I analogue and the native CAP1 sequence were comparable with each other over the entire range of peptide titration and showed half-maximal lysis at 10^{-2} μg/ml. Using the C1R-A2 cells as targets, CAP1-6D was similarly between 10^2 and 10^3 more effective in mediating lysis than CAP1 (Fig. 2*B*).

The CAP1-6D peptide was also tested using a second CEA-specific T cell line, T-Vac24 (11). This line was generated from rV-CEA postvaccination PBMCs of a different carcinoma patient by *in vitro* stimulation with the native CAP1 peptide; in contrast to predominantly CD8$^+$ T-Vac8, T-Vac24 has a high percentage of CD4$^+$CD8$^+$ double-positive cells (11). In a 4-h ^{111}In release assay using T-Vac24, CAP1-6D was slightly more effective (30% lysis) than the native CAP1 sequence (20% lysis); although the differences were not as pronounced as with T-Vac8, the increased sensitivity to the analogue was seen in three separate experiments. The possible reasons for such differences among CTLs will be discussed below. Nonetheless, the analogue peptide clearly engaged the lytic apparatus of a second CAP1-specific CTL.

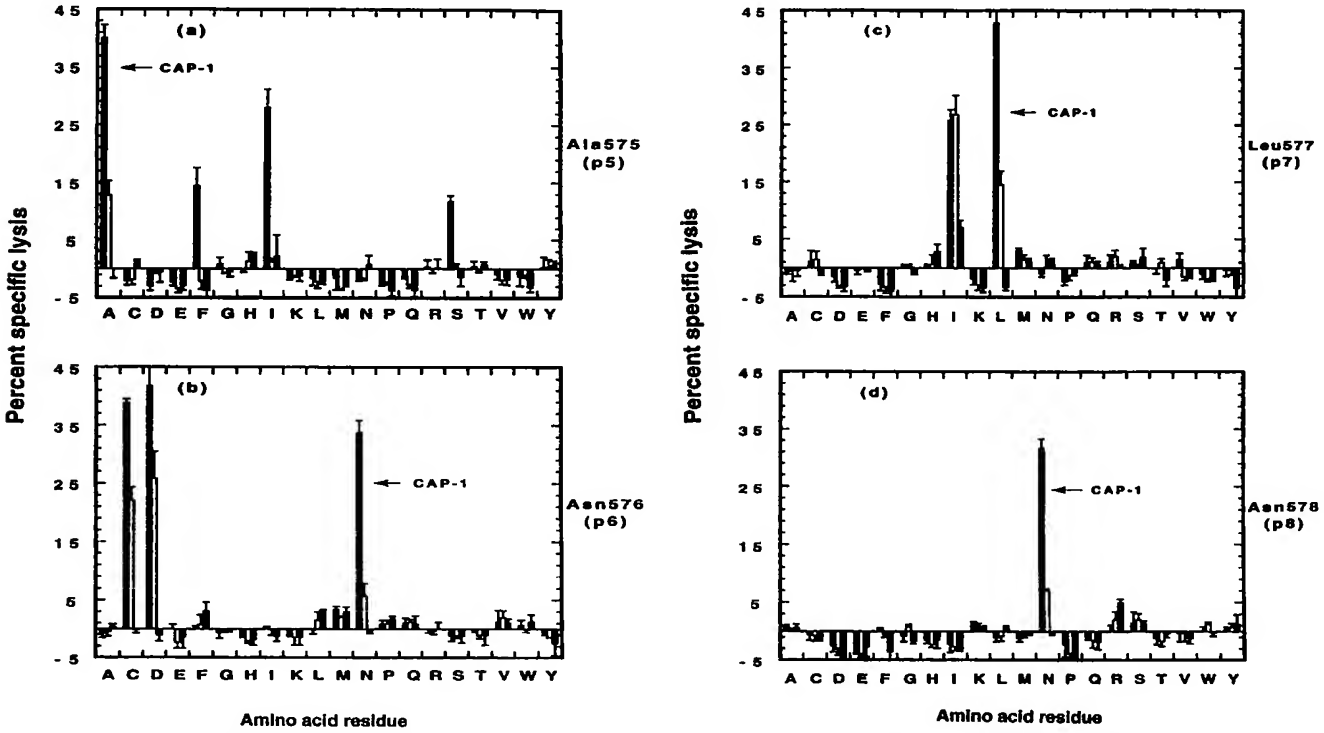

Fig. 1. Effect of single amino acid substitutions in CEA CAP1 peptide on lysis by CEA CTL-Vac8. C1R-A2 cells were labeled with ^{111}In and incubated for 1 h in round-bottom wells (2000 cells/well) with each substituted peptide at 1 (■), 0.1 (▩), and 0.01 (*hatched box*) μg/ml. T-Vac8 CTLs were added at E:T = 1.5:1, and isotope release was measured after 4 h. Spontaneous release was determined for each peptide at 1 μg/ml. All assays were performed in triplicate. *a–d*, substitutions at positions p5–p8, respectively. Amino acids are designated by the single-letter code; the amino acid encoding the native CAP1 sequence is indicated in each panel and along the right margin.

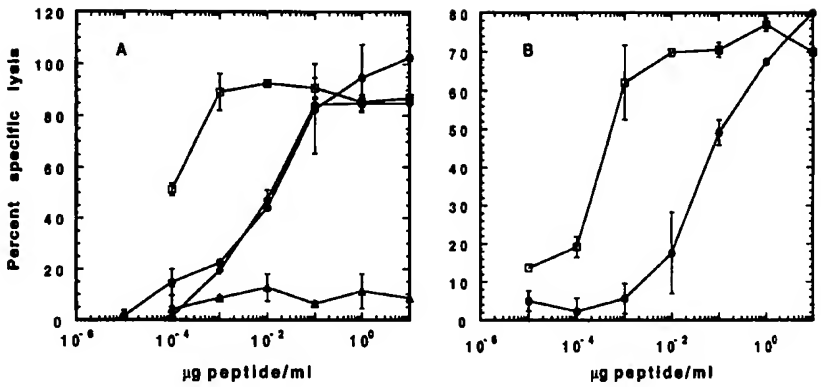

Fig. 2. CAP1 and analogues show different sensitivity to CEA CTL T-Vac8 cytotoxicity. T2 (*A*) and C1R-A2 (*B*) target cells were labeled with ^{51}Cr and incubated in round-bottom 96-well plates (10,000 cells/well) with CAP1 (●) or substituted peptides CAP1-6D (□) or CAP1-7I (◇) at the indicated concentrations. After 1 h, T-Vac8 CTLs were added at E:T = 2.5, and isotope release was determined after 4 h. All assays were done in triplicate. NCA571 (△) is a 9-mer peptide obtained after optimal alignment of CEA with the related gene NCA.

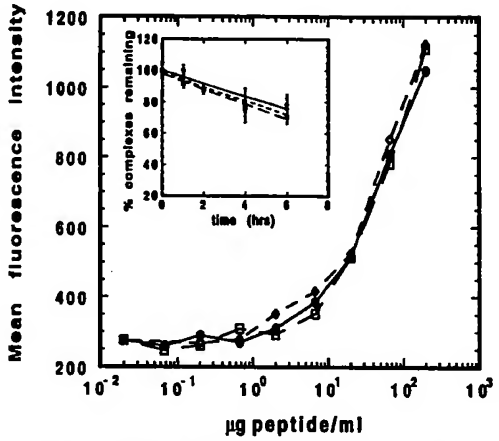

Fig. 3. Effect of single amino acid substitutions in CAP1 peptide on binding to and stability of HLA-A2 complexes. T2 cells were collected in serum-free medium and then incubated overnight (10^6 cells/well) with peptides CAP1 (●), CAP1-6D (□), or CAP1-7I (◇) at the indicated concentrations. Cells were collected and assayed for cell surface expression of functional HLA-A2 molecules by staining with conformation-sensitive mAb BB7.2, HLA-specific antibody W6/32 (data not shown), and isotype control antibody MOPC-195 (data not shown). MFI was determined on a live, gated cell population. *Insert*, cells were incubated with peptide at 100 µg/ml overnight and then washed free of unbound peptide and incubated at 37°C. At the indicated times, cells were stained for the presence of cell surface peptide-HLA-A2 complexes. *Error bars*, SE for two experiments.

Analogues and Native Peptide Show Identical Presentation by HLA-A2. The increased effectiveness of CAP1-6D in CTL assays could be due to better presentation by the target. The most active CAP1 analogues were tested for binding to HLA-A2 by measuring cell surface HLA-A2 in the transport-defective human cell line T2. When compared over a 4-log range of concentrations, native CAP1 and the two analogues CAP1-6D and CAP1-7I all presented equally on T2 cells (Fig. 3). In addition, dissociation experiments indicate that the HLA-A2 complexes that form with the three peptides show no appreciable differences in stability (Fig. 3, *insert*). When peptide-pulsed T2 cells were washed free of unbound peptide, the half-lives of cell surface peptide-A2 complexes were 12.5 (CAP1), 9.7 (CAP1-6D), and 10.8 h (CAP1-7I). If anything, the complex formed with the agonist peptide seems slightly less stable. Because there are no differences in binding to HLA-A2, the improved effectiveness of CAP1-6D in the CTL assays seems to be due to better engagement by the TCR, a behavior characteristic of an enhancer agonist peptide. The binding studies shown in Fig. 3 also demonstrate that the over 100-fold difference observed in lysis of T2 cells by CAP1-6D *versus* CAP1 (Fig. 2) is not due to differences in the solubility of the two peptides, because both bound to HLA-class I identically, using identical conditions and the same T2 cell line.

Human CTLs Generated with CAP1-6D Also Recognize Native CAP1. The CAP1-6D agonist might be useful in both experimental and clinical applications if it can stimulate growth of CEA-specific CTLs from patients with established carcinomas. In one experiment, post rV-CEA immunization PBMCs from cancer patient Vac8 (the same rV-CEA patient from whom T-Vac8 CTLs were established) were stimulated *in vitro* with CAP1-6D, and after six rounds of stimulation, they were assayed for CTL activity against targets coated with CAP1 or CAP1-6D. This new line demonstrated peptide-dependent cytotoxic activity against target cells coated with either CAP1-6D or native CAP1 (Table 1).

Postimmunization PBMCs from patients Vac8 and Vac24 were already shown to produce CTL activity when stimulated with CAP1, whereas preimmunization PBMCs were negative (11, 34). Moreover, previous attempts to stimulate CTL activity from healthy, nonimmunized donors with the CAP1 peptide were unsuccessful. To test whether the agonist peptide is indeed more immunogenic than native CAP1, we attempted to generate CTLs from two healthy, nonimmunized donors comparing CAP1 and CAP1-6D at the same time, using PBMCs from the same donors and identical methodology (see "Materials and Methods"). HLA-$A2^+$ PBMCs from both apparently healthy individuals were stimulated *in vitro* with either CAP1 or the CAP1-6D agonist. After four cycles of *in vitro* stimulation, cell lines were assayed for specificity against C1R-A2 cells pulsed with either CAP1 or CAP1-6D. Whereas stimulations with CAP1 or the CAP1-6D peptide produced T-cell lines, peptide-specific lysis was only obtained in the lines generated with CAP1-6D from both donors and not when using CAP1. Two independent T-cell lines from different donors were derived using CAP1-6D and designated T-N1 and T-N2 (Fig. 4, *A* and *B*, respectively). Both CTL lines lyse C1R-A2 targets pulsed with native CAP1 peptide. However, more efficient lysis is obtained using the CAP1-6D agonist. T-N1 CTL recognizes CAP1-6D at lower peptide concentrations than CAP1 (Fig. 4*A*), and T-N2 recognizes the agonist 100-fold better than CAP1 (Fig. 4*B*). In contrast, attempts to generate CTL cell lines from normal donors by

Table 1 *CTLs generated by stimulation with the CAP1-6D analogue from PBMCs of an HLA-A2 patient immunized with rV-CEA*

T cells were assayed after six *in vitro* stimulations. Cytotoxic activity was determined in a 4-h release assay with peptide at 25 µg/ml. C1RA2 cells were used as targets.

	% Lysis		
E:T ratio	No peptide	CAP1	CAP1-6D
25:1	10%	41%	40%
6.25:1	0.5%	38%	46%

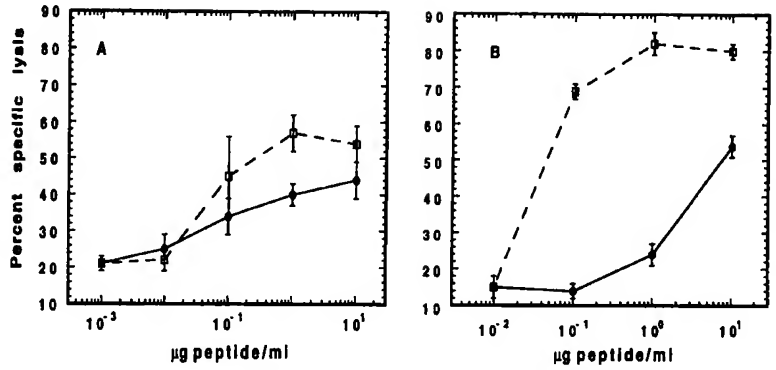

Fig. 4. CTLs generated from apparently healthy individuals with CAP1-6D recognize CAP1 and CAP1-6D. CTL lines designated T-N1 and T-N2 were generated with CAP1-6D and assayed for peptide specificity. T-N1 was assayed after five cycles of stimulation at an E:T ratio of 20:1 (*A*). T-N2 was assayed after 10 cycles at an E:T ratio of 15:1 (*B*). ^{51}Cr-labeled C1R-A2 targets (5000 cells/well) were incubated with the indicated amount of CAP1 (●) or CAP1-6D (□) peptide. After 4 h, the amount of isotope release was determined in a gamma counter. Values were determined from triplicate cultures.

stimulation with CAP1 resulted in cell lines with no peptide-dependent lysis and loss of the cell lines in early stimulation cycles. Because so few CAP1-derived cells were available due to poor growth, phenotypic analysis of these cells was not possible. However, after 14 cycles of stimulation of PBMCs from donor 1 with CAP1-6D, the T-N1 cell line was shown to be 86% $CD8^+$ and 0.3% $CD4^+$; likewise, after 15 cycles of stimulation of PBMCs from donor 2 with CAP1-6D, phenotypic analysis of the T-N2 line revealed 98% $CD8^+$ and 0.5% $CD4^+$. Thus, the attempts to generate T-cell lines using the two peptides (CAP1 and CAP1-6D) demonstrated the ability of CAP1-6D to act as an agonist not only at the effector stage, in the lysis of targets, but also in selecting T cells that are presumably in low precursor frequencies.

To determine whether CTLs established with the agonist could be maintained on the native CAP1 sequence, T-N1 was cultured for five cycles as described using CAP1-6D and then divided into duplicate cultures maintained on the agonist or on CAP1. T-N1 continued to grow when stimulated with either peptide and responded to both peptides in CTL assays (data not shown). Phenotypic analysis of the TCR usage in T-N1 indicates that the majority of cells (71%) use Vβ12, with a minor population that uses Vβ5.3 (Table 2). The same pattern of TCR Vβ usage was observed after switching the cells to CAP1 for five more stimulation cycles. This Vβ usage pattern was distinct from that of T-Vac8, which is predominantly Vβ1, 8, and 17.[5] These data indicate that the agonist can select T cells that are probably in low precursor frequency, but that once selected, such CTLs could be maintained with the native CAP1.

Table 2 *TCR usage of a CTL line established on CAP1-6D agonist*

	T-N1[a]		T-N1[b]	
TCR usage[c]	% Positive	MFI	% Positive	MFI
Vβ12	71	83	70	83
Vβ5.3	18	47	20	57
Vβ3.1	6	48	8	46
Vβ8	3	30	6	26
Vβ13.6	2	19	3	39
Vβ12.1	3	43	3	40

[a] CTL line selected and maintained on agonist peptide CAP1-6D as described in "Materials and Methods."
[b] CTL line selected on agonist CAP1-6D for 5 stimulation cycles and maintained on CAP1 for an additional 10 cycles.
[c] Determined by FACS analysis using a panel of 19 Vβ and 2 Vα antibodies (see "Materials and Methods"). Only positively staining antibodies are shown.

CTLs Generated with CAP1-6D Specifically Lyse CEA^+, $HLA\text{-}A2^+$ Tumor Cells. Studies were conducted to determine the ability of CTLs generated with the enhancer agonist to lyse human tumor cells endogenously expressing CEA. T-N1 and T-N2 were tested against a panel of tumor cells that are $CEA^+/A2^+$ (SW480 and SW1463), $CEA^+/A2^-$ (SW1116), or $CEA^-/A2^+$ (CaOV3 and SK-mel24). A T-cell line (T-N2) from the normal donor was tested for the ability to lyse tumor targets endogenously expressing CEA. T-N2 CTLs generated with the agonist lysed tumor cells expressing both CEA and HLA-A2 while exhibiting no titratable lysis of $CEA^-/A2^+$ SKmel24 melanoma cells (Fig. 5*A*). No significant lysis of K562 was observed. In contrast, cell lines generated by stimulation with native CAP1 showed no detectable antitumor lytic activity (Fig. 5*B*). The HLA-A2.1 restriction of the T-N2 response to CEA-positive tumor targets was further demonstrated by the specific lysis of a CEA-positive HLA-A2.1-negative tumor cell, SW837, after infection with a vaccinia-A2.1 construct (rV-A2.1). No lysis was observed when SW837 targets were infected with the control wild-type vaccinia without the A2.1 transgene (Fig. 6).

The ability of a CTL cell line (T-N1) derived from a second donor to kill carcinoma targets expressing endogenous CEA is shown in Fig.

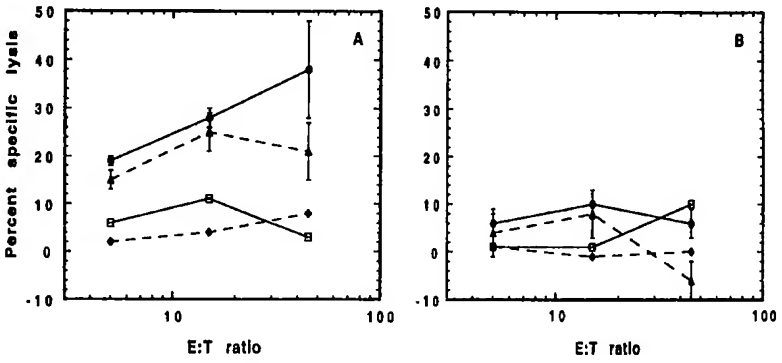

Fig. 5. CAP1-6D- but not CAP1-generated T-cell lines from apparently healthy donors recognize tumor cells expressing endogenous CEA. CAP1-6D-generated T-N2 CTLs (*A*) and T cells generated with native CAP1 (*B*) were assayed after nine cycles of *in vitro* stimulation against tumor targets SW480 (CEA^+, $HLA\text{-}A2^+$; ●) and SW1463 (CEA^+, $HLA\text{-}A2^+$; ▲), SKmel24 (CEA^-, $-A2^+$; □), and K562 (◇). Tumor cells were cultured for 72 h in the presence of IFN-γ to up-regulate HLA. Cells were trypsinized and labeled with ^{51}Cr and incubated (5,000 cells/well) with T-N2 CTLs at increasing E:T ratios. Cultures were incubated for 4 h, and the amount of isotope release was determined in a gamma counter. Values were determined from triplicate cultures.

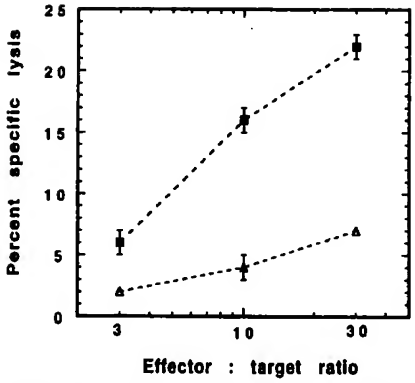

Fig. 6. MHC-class I A2.1 restriction of CTL line (T-N2) derived from CAP1-6D agonist. CTL line T-N2 was used as an effector for the human colon carcinoma SW837 target cell. SW837 is CEA positive and HLA-A2.1 negative. SW837 cells were infected at a multiplicity of infection of 10:1 with either a recombinant vaccinia containing the A2.1 transgene (■) or wild-type vaccinia (△).

7. T-N1 specifically lysed SW480 tumor cells. This is dramatically enhanced to 79% lysis by pretreatment of the tumor cells with IFN-γ, a treatment that increases the cell surface density of both HLA-A2 and CEA (data not shown). The specificity of T-N1 killing is demonstrated by its inability to lyse $CEA^-/A2^+$ tumors such as the ovarian-derived tumor cell line CaOV3, the melanoma tumor cell line SK-mel24, or the natural killer target K562 (data not shown). Finally, restriction by HLA-A2 is demonstrated by the failure of T-N1 to lyse $CEA^+/A2^-$ SW1116 tumor cells even after IFN-γ treatment (Fig. 7).

DISCUSSION

We recently reported (11) evidence of CTL responses to CEA in patients immunized with rV-CEA. The 9-mer peptide CAP1 was used to expand CTLs *in vitro* because of: (*a*) its strong binding to HLA-A2; and (*b*) its nonidentity to other members of the CEA gene family expressed on normal tissues. CTLs were generated from postimmunization PBMCs of patients, whereas preimmunization blood of the same patients failed to proliferate. In addition, CAP1-pulsed dendritic cells stimulated *in vitro* growth of HLA-A2-restricted CTLs from peripheral blood of unimmunized cancer patients (12). Finally, when CTLs were generated *in vitro* by stimulation with dendritic cells encoding full-length CEA mRNA, cytotoxicity against CAP1 was higher than activity against six other HLA-A2 binding CEA peptides. Such results encourage the notion that CAP1 is an immunodominant epitope of the CEA molecule.

The studies reported here were designed to improve the immunogenicity of the CAP1 peptide by introducing amino acid substitutions at nonanchor positions. When using T-Vac8 CTL as an effector, the analogue CAP1-6D sensitized target cells for lysis far better than CAP1 itself. Additional studies showed that cytolytic activity of a second HLA-A2-restricted CAP1-specific CTL, T-Vac24, was as good or greater with CAP1-6D than it was with CAP1. These demonstrations of enhanced reactivity could not be explained by improved presentation by class I MHC. Finally, CAP1-6D could be used to stimulate CTLs *in vitro* from PBMCs of both carcinoma patients and normal donors. Several previous attempts to stimulate anti-CAP1 CTLs from normal donors using this same methodology have been unsuccessful. In the present study, we compared the stimulation of two normal donors with native CAP1 and CAP1-6D; once again, stimulation with the native sequence failed to produce specific cytotoxic activity. In contrast, stimulation with CAP1-6D produced several CTLs with specific anti-CAP1 peptide reactivity as well as antitumor reactivity. These findings indicate that the analogue peptide CAP1-6D is capable of selecting a population of CAP1-specific human CTLs more efficiently than native CAP1. Such an agonist might find applications in the design of T cell-directed vaccines against CEA-expressing carcinoma.

A more immediate application might be in the more efficient generation and expansion of tumor-specific T cells for adoptive immunotherapy. In recent years, much progress has been achieved in characterizing the TAA peptides that can be presented to CTLs by class I HLA antigens. In instances where mutations generate neo-antigens, such as point-mutated ras (35, 36), p53 (37, 38), or β-catenin (39), vaccination strategies target the novel sequence under the assumption that the immune system is not tolerant to an antigen it has never seen. More recently, it has been proposed that neo-antigens may also arise through post-translational deamidations (29, 40). However, in many instances, the intended targets of tumor therapy are not neo-antigens but rather normal oncofetal or differentiation antigens that are overexpressed or ectopically expressed by malignant cells. Such is the case for CEA (41). In such situations, models invoking tolerance predict that the immune system has encountered these antigens and is less able to respond to them. This classical picture has been challenged in recent years by numerous reports of immunity elicited to overexpressed differentiation antigens, oncogenes, and tumor suppressor genes (37, 38, 42–44). Nonetheless, it is often experimentally difficult to generate and expand T cells with desired antitumor activity, and it is therefore desirable to devise new strategies for generating CTLs.

Some class II binding peptides have been described in which substitutions enhance responses of murine and human Th clones without increasing the binding to class II antigens (29, 45–47). Among human class I peptides, however, the only substitutions de-

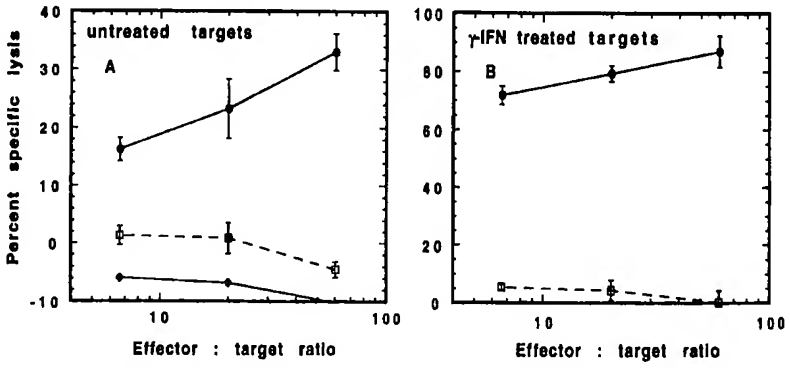

Fig. 7. CTLs generated with CAP1-6D lyse CEA-positive HLA-A2-positive tumors: effect of IFN-γ. The T-N1 CTLs generated with CAP1-6D were assayed against various tumor cell lines: SW480 (CEA^+ and $HLA\text{-}A2^+$; ●), SW1116 (CEA^+ but $HLA\text{-}A2^-$; □), and CaOV3 (CEA^- but $HLA\text{-}A2^+$; ◇). Tumor cells were cultured for 72 h in the absence (*A*) or presence (*B*) of IFN-γ, trypsinized, labeled with ^{51}Cr, and then incubated (5,000 cells/well) with T-N1 CTL at increasing E:T ratios. Cultures were incubated for 4 h, and the amount of isotope release was determined in a gamma counter. Values were determined from triplicate cultures.

scribed for the generation of CTLs were those that increase binding to HLA (17–20). The substitutions in those studies were directed to residues at the primary or secondary anchor positions that define the binding motifs to class I MHC antigens. Even substitutions directed to a nonanchor position (19) achieved their enhancing effect by increasing binding to HLA-A2. The analogue CAP1-6D in the present report represents what appears to be a different class of substituted CTL peptides, agonists that enhance recognition of the peptide-MHC ligand by the TCR and produce greater effector function without increases in binding. To our knowledge, this is the first such enhancer agonist peptide described for a human CTL.

The increased lytic susceptibility of targets in the presence of CAP1-6D is unlikely to be due to better antigen presentation. Binding experiments show that HLA-A2 presents the native CAP1 and the analogues CAP1-6D and CAP1-7I approximately equally. Another possibility is that CAP1-6D shows increased activity because it is presented by more than one allele and T-Vac8 is promiscuous toward peptide-MHC complexes. However, T-Vac8, T-Vac24, and CTLs derived from nonimmunized patients showed better lysis with CAP1-6D. Because HLA-A2 is the only class I MHC on the targets used, the improved lysis cannot be accounted for by recruitment of another class I MHC.

Because anti-CAP1 CTLs from multiple donors demonstrate agonist cross-reactivity, it is possible that CAP1-6D could be used to stimulate the growth of CTLs from numerous HLA-A2 individuals. We are encouraged by the quite distinct differences between T-Vac8 and T-Vac24 in magnitude of response to the agonist; this implies that each effector uses different TCR gene segments and that, nonetheless, they can recognize both the native sequence and the CAP1-6D substitution. The ability of CAP1-6D to act as an agonist with T cells expressing different TCRs clearly magnifies its therapeutic potential. It can therefore be speculated that stimulation with the agonist could generate T cells that might recognize the normal sequence in nonimmunized individuals. Such individuals have presumably never encountered the modified sequence, and because the agonist is more efficient at triggering a T-cell response, such agonists might be capable of selecting CTLs more readily than immunogens based on the native sequence.

For peptide-derived CTLs to be useful therapeutic reagents, it is essential to demonstrate that they can lyse tumor cells that express endogenous antigen (48, 49). Previously (11), we had shown that tumor cells process CEA and present antigens recognized by CTLs generated by stimulation with CAP1. In the present study, CTLs grown from the normal donors by stimulation with CAP1-6D are also capable of recognizing allogeneic CEA-positive HLA-A2-positive tumor cells. These T cells fail to recognize HLA-A2-negative tumor cells or HLA-A2-positive cells that lack CEA expression.

We have also shown that CTLs selected with the CAP1-6D agonist can be maintained subsequently by stimulation with the native CAP1 sequence. This is an important finding, because CTLs in patients, whether established *in vivo* through active immunization or transferred adoptively after *ex vivo* expansion, will likely only encounter the native sequence. It could thus be hypothesized that the CTLs could be maintained over an extended duration *in vivo*.

One of the original reasons for selecting and testing CAP1 was its nonidentity with other reported sequences in the human genome. It was therefore predicted that any immune responses attained would be unlikely to damage normal tissues bearing other antigens. For this reason, a similar search of protein databases was undertaken for the peptides CAP1-6D and CAP1-7I and revealed that they are not reported as human sequences elsewhere in the GenBank (Genetics Computer Group, Madison, WI).

Two recent reports suggest that modified asparagine residues might enhance the immunogenicity of class I MHC peptides. Skipper *et al.* (40) used CTLs generated in mixed lymphocyte tumor cell cultures to identify antigens in extracts of melanoma cells. One antigenic peptide was identical at eight of nine positions to a sequence from tyrosinase, with an asparagine to aspartic acid replacement at position 3. When tested using synthetic peptides, the CTLs were more active against the aspartic acid peptide than against the peptide containing the genetically predicted asparagine. These authors speculate that post-translational deamidations can generate antigenic peptides from normal differentiation antigens. Recently, Chen *et al.* (50) reported generating murine CTLs to a stabilized succinimide derivative of an asparagine-containing antigenic peptide. Although these CTLs could kill targets pulsed with the natural asparagine peptide, they did so with less sensitivity. They raise the possibility that deamidation of proteins *in vivo* and *in vitro* can produce transient succinimide intermediates that represent altered self-ligands capable of eliciting an immune response. At the other extreme, Kersh and Allen (51) replaced a TCR contact asparagine with aspartic acid in a hemoglobin peptide and abolished responsiveness to a murine Th clone. Presently, we cannot exclude the possibility that the enhanced reactivity of CAP1-6D is due to deamidation of the native sequence, which in turn primes the response that we detect with CAP1. However, our repeated inability to raise anti-CAP1 CTLs from preimmunized PBMCs of the same patients from whom we generated postimmunization CTLs argues against this. Also, putative deamidations could not account for the recognition of other analogues such as CAP1-6C or CAP1-7I by T-Vac8 CTLs. Instead, it seems more reasonable that TCRs from both T-Vac8 and T-Vac24, as well as the new lines described here, can recognize some deviation from the native CAP1 sequence.

In summary, synthesis of analogues of an immunodominant CEA peptide with amino acid substitutions at positions predicted to potentially interact with the TCR allowed us to identify an enhancer agonist. This agonist was recognized by two different CEA CTLs and increases the activity of one of them by 2–3 orders of magnitude. The agonist was also able to stimulate the growth of CTLs from peripheral blood of nonimmunized normal donors with far greater facility than the native peptide sequence. Most important, the CTL generated using the enhancer agonist was able to recognize and lyse targets presenting the native sequence, including tumor cell lines expressing endogenous CEA. We believe that characterization of this enhancer agonist peptide may permit more aggressive antitumor immunotherapies when used as an immunogen *in vivo* or for the *ex vivo* expansion of autologous antitumor CTLs. The synthetic approach used here may also prove useful in improving immunogenicity of other peptide CTL epitopes.

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The Ability of Variant Peptides to Reverse the Nonresponsiveness of T Lymphocytes to the Wild-Type Sequence $p53_{264-272}$ Epitope[1]

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Recently, we observed that CTL specific for the wild-type (wt) sequence $p53_{264-272}$ peptide could only be expanded ex vivo from PBMC of a subset of the HLA-A2.1$^+$ normal donors or cancer patients tested. Surprisingly, the tumors of the responsive patients expressed normal levels of wt p53 and could be considered unlikely to present this epitope. In contrast, tumors of nonresponsive patients accumulated mutant p53 and were more likely to present this epitope. We sought to increase the responsive rate to the wt $p53_{264-272}$ peptide of PBMC obtained from normal donors and patients by identifying more immunogenic variants of this peptide. Two such variants were generated by amino acid exchanges at positions 6 (6T) and 7 (7W) of the peptide. These variants were capable of inducing T cells from PBMC of nonresponsive donors that recognized the parental peptide either pulsed onto target cells or naturally presented by tumors. TCR Vβ analysis of two T cell lines isolated from bulk populations of effectors reactive against the wt $p53_{264-272}$ peptide, using either the parental or the 7W variant peptide, indicated that these T cells were expressing identical TCR Vβ13.6/complementarity-determining region 3/J region sequences. This finding confirms the heteroclitic nature of at least one of the variant peptides identified in this study. The use of variant peptides of the wt $p53_{264-272}$ epitope represents a promising approach to overcoming the nonresponsiveness of certain cancer patients to this self epitope, thereby enhancing its potential use in tumor vaccines for appropriately selected cancer patients. *The Journal of Immunology,* **2002, 168: 1338–1347.**

Genetic alterations in p53 occur in a wide range of human tumors, including oral squamous cell carcinomas (OSCC)[3] (1). The most common type of genetic alteration in p53 involves a missense mutation that is usually accompanied by accumulation of the altered molecules in the cytosol of tumor cells. Initially, the effort to develop p53-based vaccines focused on these missense mutations, which are tumor specific in nature. However, missense mutations have limited clinical usefulness, because of the requirement that they occur within or create epitopes that could be presented by MHC molecules expressed by the individual patient. On the other hand, the majority of p53 epitopes derived from these altered p53 molecules would be wild type in sequence, representing a new class of tumor-associated self Ags that are candidates for use in the development of broadly applicable cancer vaccines (1–5).

To date, five MHC class I-restricted, naturally presented human wild-type (wt) sequence p53 epitopes have been identified. They have been shown to be able to induce epitope-specific CTL from PBMC obtained from healthy individuals (1, 6–11). The $p53_{125-134}$ epitope is HLA-A24 restricted (11), while the other four, $p53_{65-73}$, $p53_{149-157}$, $p53_{217-225}$, and $p53_{264-272}$, are HLA-A2.1 restricted. Among these, the wt $p53_{264-272}$ peptide has been the most intensively investigated (1, 2, 6–8, 12).

The potential of wt p53 epitopes as targets for immunotherapy, however, remains uncertain due to the several critical concerns related to immunological recognition of this truly self tumor Ag. Using HLA-2.1-transgenic wt ($p53^{+/+}$) and $p53^{null}$ ($p53^{-/-}$) mice, Sherman and colleagues (13–15) have demonstrated that the CTL repertoire available for wt p53 self epitopes in $p53^{+/+}$ mice is limited to intermediate affinity T cells, because the higher affinity CTL are either deleted or tolerized. Apparently, this situation occurs in humans as well, as only CTL with intermediate affinity for the wt $p53_{264-272}$ epitope have been generated to date from PBMC obtained from normal donors as well as cancer patients (7, 12). This observation raises the question of whether such CTL are potent enough to be effective in tumor eradication.

Another concern relates to our experience that PBMC obtained from only some HLA-A2.1$^+$ healthy donors and patients with OSCC were responsive to in vitro stimulation (IVS) with the wt $p53_{264-272}$ peptide pulsed onto autologous dendritic cells (DC) (7, 12). Furthermore, CTL reactive against this epitope could only be generated from T cell precursors in PBMC of patients whose tumors were not likely to present this epitope. The analysis of these

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[3] Abbreviations used in this paper: OSCC, oral squamous cell carcinoma; APL, altered peptide ligand; CDR, complementarity-determining region; DC, dendritic cell; IVS, in vitro stimulation; wt, wild type.

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tumors indicated no accumulation of p53 or accumulation of mutant p53 with a missense mutation at codon 273, a site known to block processing of the wt $p53_{264-272}$ epitope (16). In contrast, PBMC obtained from patients with tumors considered capable of presenting the wt $p53_{264-272}$ epitope (i.e., tumors that accumulate mutant p53) were nonresponsive to IVS with wt $p53_{264-272}$-pulsed autologous DC. These findings have led us to conclude that CTL specific for the wt $p53_{264-272}$ epitope might play a role in the outgrowth of epitope-loss tumor cells, which are able to escape from the host immune system. This conclusion was further strengthened by the results of a recently completed study in our laboratories that used tetrameric peptide/MHC class I complexes to determine frequencies and characteristics of the $p53_{264-272}$-specific CTL in unstimulated PBMC obtained from 30 OSCC patients and 31 normal donors (data not shown).[4]

Because these observations suggest that it may be possible to accurately predict ex vivo the responsiveness of cancer patients to immunotherapy targeting this epitope, we felt that a means of circumventing the nonresponsiveness of individuals needed to be investigated to proceed with the development of wt p53-based vaccines. One solution is to identify a heteroclitic peptide or, in more precise terms, an altered peptide ligand (APL) with enhanced functional activity relative to the parental wt $p53_{264-272}$ peptide. By substituting amino acids at various positions of an epitope that contact MHC class I and/or TCR, an array of APL with biological potencies higher than those of the parental epitopes has been identified for various antigenic determinants (17–26). In applying this strategy to the wt $p53_{264-272}$ epitope, we anticipated that an APL might induce CTL-mediated responses that cross-react with the parental epitope and that these CTLs also might demonstrate enhanced avidities relative to CTLs induced by the parental peptide. Most importantly, we sought to determine whether an APL would be able to induce anti-wt $p53_{264-272}$ CTL from PBMC that were nonresponsive to the parental peptide, particularly the PBMC obtained from patients whose tumors accumulate mutant p53 and are considered to have the potential to present this epitope.

Materials and Methods

Cell lines and cell culture

The following HLA-$A2^+$ OSCC cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA): SCC-4 and SCC-9. The SCC-4 cell line expresses and accumulates p53 expressing a missense mutation at codon 151 but does not present the wt $p53_{264-272}$ epitope (6). The SCC-9 cell line expresses an altered p53 molecule with a deletion of codons 274–285. It does not accumulate p53 molecules, yet presents the wt $p53_{264-272}$ epitope. In addition, the $p53^{null}$ osteosarcoma cell line, SaOS-2, was obtained from ATCC. The cloned $p53^+$ cell line, SaOS-2Cl3, was derived by transduction of SaOS-2 cells with a p53 cDNA expressing a missense mutation in codon 143 (7). The HLA-$A2^+$ OSCC cell line PCI-13 has been described previously (27). It expresses a p53 missense mutation in codon 286 (Glu to Lys) and presents the wt $p53_{264-272}$ epitope. Tumor cells were cultured in plastic culture flasks (Costar, Cambridge, CA) under standard conditions (37°C, 5% CO_2 in a fully humidified atmosphere) in complete medium consisting of DMEM (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 IU/ml penicillin (all from Life Technologies). The T2 cell line was also obtained from ATCC and maintained in RPMI 1640 (Life Technologies) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics. The cultures were routinely tested and found to be free of mycoplasma contamination (Gen-Probe, San Diego, CA).

[4] T. K. Hoffmann, A. Donnenberg, S. Finkelstein, K. Chikamatsu, V. Donnenberg, U. Friebe, E. Appella, A. B. DeLeo, and T. L. Whiteside. Frequencies of $tetramer^+$ T cells specific for the wild type sequence $p53_{264-272}$ peptide in the circulation of patients with head and neck cancer. *Submitted for publication.*

Peptides

The CTL-defined, HLA-A2.1-binding peptide, LLGRNSFEV (1), corresponding to wt $p53_{264-272}$, as well as single amino acid exchange variants of this peptide were synthesized by standard *N*-(9-fluorenyl)methoxycarbonyl methology. Peptides were purified by reversed-phase HPLC, and their amino acid sequence was confirmed by mass spectrometry analysis. All peptides were dissolved in DMSO (Fisher Scientific, Pittsburgh, PA) at 1 mg/ml and diluted with PBS just before use. The 19 variant peptides contain single amino acid exchanges with a bias toward retention of a high degree of similarity to the central region of the parental peptide. The variant peptides are designated 1E, 1F, 1V, 3L, 3F, 3W, 4K, 4L, 5K, 5L, 6G, 6T, 6Y, 7L, 7P, 7Y, 7W, 8A, and 8Y, in which numbers denote the position within the parental sequence and letters refer to exchanged amino acids.

MHC stabilization assay

T2 cells were incubated overnight at room temperature before use in this assay. Cells were washed and incubated at a cell density of 2×10^5/0.2 ml of complete medium with various peptides at final concentrations of 1×10^{-5}–1×10^{-10} M for 3 h at room temperature, followed by a 3-h incubation period at 37°C. After washing with PBS, cells were incubated at 4°C for 30 min with anti-HLA class I mAb, W6/32 (HB95; ATCC), and then with FITC-conjugated goat anti-mouse Ig (Caltag Laboratories, Burlingame, CA) as a secondary Ab. Fluorescence of viable T2 cells was measured at 488 nm in a FACScan flow cytometer (BD Biosciences, San Jose, CA), and the level of MHC class I expression was determined by evaluating the mean fluorescence intensity of stained T2 cells. Cells incubated either at room temperature or 37°C in the absence of peptide served as controls.

Generation of anti-p53 CTL with peptide-pulsed autologous DC

Peripheral blood or leukapheresis products were obtained from previously studied HLA-$A2.1^+$ individuals: seven normal donors and six OSCC patients (12). PBMC were isolated by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ). The study was approved by the Institutional Review Board at the University of Pittsburgh, and written informed consent was obtained from each individual donating peripheral blood. PBMC were phenotyped for HLA-A2 expression by flow cytometry, using anti-HLA-A2 mAb, BB7.2 (HB82; ATCC), and a mouse IgG isotype as a control. The verification of the A0201 subtype was performed using PCR with sequence-specific primers, as previously described (12).

Human DC were generated from PBMC according to a modification of the method of Sallusto and Lanzavecchia (28), as described by us earlier (7). DC were harvested on day 6, phenotyped by flow cytometry, and then resuspended in AIM-V medium (2×10^6 cells/ml) containing 10 μg/ml peptide and incubated at 37°C for 4 h. The peptide-pulsed DC were then cocultured with autologous PBMC in 24-well tissue culture plates (Costar) in a final volume of 2 ml/well AIM-V medium supplemented with 10% (v/v) human AB serum (Pel-Freez Biologicals, Brown Deer, WI) and 25 ng/ml IL-7 (Genzyme, Cambridge, MA) for the first 72 h and, additionally, with 20 IU/ml IL-2 (Chiron-Cetus, Emeryville, CA) for the remaining time in culture. The lymphocytes were restimulated 1 wk later with peptide-pulsed autologous DC. Irradiated (3000 rad) autologous PBMC were used as APC after the third round of restimulations. Microcultures of CTL lines recognizing the wt $p53_{264-272}$ or 7W peptide were isolated from bulk populations of effectors by limiting dilution (1 cell/well/96-well plates), and the lines were maintained in cytokine-supplemented media plus peptide-pulsed APC, as previously described (7). Specificities of generated T cells were determined using one or more of a panel of assays detailed below. The TCR Vβ expression on T cells in bulk CTL populations and cell lines derived from them was done using the IOTest β Mark TCR Vβ Repertoire kit (Beckman Coulter, San Diego, CA).

ELISPOT assay for IFN-γ

The ELISPOT assay was performed in 96-well plates with nitrocellulose membrane inserts (Millipore, Bedford, MA), as previously described by us (29). The capture and detection anti-IFN-γ mAb were purchased from Mabtech (Nacka, Sweden). The spots were counted by computer-assisted image analysis (ELISPOT 4.14.3; Zeiss, Jena, Germany). For Ab-blocking experiments, target cells were preincubated with anti-HLA class I mAb for 30 min. Cryopreserved aliquots of PBMC obtained from a normal donor were thawed and, after stimulation with PMA (1 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich, St. Louis, MO), were used as a positive control for each assay.

The interassay reproducibility of the assay was acceptable with a coefficient of variation = 15% (n = 30).

Cytotoxicity assay

The 4-h ^{51}Cr release assay was performed at various E:T ratios, as previously described (7). Briefly, sensitized targets were labeled with ^{51}Cr for 45 min at 37°C, washed, and added to wells of 96-well plates (1×10^4 cells/well). Effector T cells were then added to give various E:T ratios. When Ab-blocking experiments were performed, target cells were incubated with anti-HLA class I mAb or the anti-HLA-DR mAb, L243 (HB55; ATCC),for 30 min before adding effector cells. The percentage of specific lysis was calculated according to the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental cpm} - \text{control cpm}}{\text{maximal cpm} - \text{control cpm}} \times 100$$

Flow cytometry analysis using HLA-A2.1/peptide tetrameric complexes (tetramer)

The streptavidin-PE-labeled tetramers used in this study were obtained from the tetramer core facility of the National Institute of Allergy and Infectious Disease (Atlanta, GA). Three-color flow cytometry assays (FACScan; BD Biosciences) were performed with PerCP anti-CD3, FITC anti-CD8, and PE-tetramer. The specificity of the HLA-A2.1/$p53_{264-272}$ tetramer was confirmed by its staining of a CTL line specific for this p53 epitope and by the lack of staining of irrelevant CTL or HLA-A2$^-$ PBMC of healthy donors, as previously described (30). The additional PE-conjugated HLA-A2.1/tetramer used in this study contained the 7W variant peptide. Generally, 75,000 events per sample were collected progressively after live gating on lymphocytes by forward and side scatter.

TCR and CDR3 spectratyping

RNA was extracted from $p53_{264-272}$-specific CTL lines generated using parental or the 7W variant peptide, followed by reverse transcription into cDNA, as previously described (31). Screening for expression of TCR V chains was performed using the primers described by Puisieux et al. (31) for TCR Vβ amplification, followed by a runoff reaction with fluorophore-labeled primers specific for the C region of the TCRβ (5′-TGTGCAC CTCCTTCCCATTCACC) chain. Labeled runoff products were subjected to DNA fragment analysis, as described (32). Finally, amplified products were directly subjected to DNA sequence analysis using ABI 310 sequencer (PerkinElmer, Weiterstadt, Germany).

Statistical analysis

A two-tailed Wilcoxon rank sum test was performed to analyze ELISPOT data. An unpaired two-tailed Student's t test was used to interpret differences in CTL reactivities against different target cells and in the presence of blocking Ab in cytotoxic assays, and differences between the number of spots obtained from T cells incubated with T2 cells pulsed with relevant p53 peptides vs that obtained using T2 cells pulsed with the irrelevant gp100 peptide in ELISPOT assays. Differences were considered significant when $p < 0.05$.

Results

Selection of variant $p53_{264-272}$ peptides recognized by anti-$p53_{264-272}$ CTL

Because the parental peptide binds efficiently to HLA-A2.1 molecules, all of the APL considered in this study represent single amino acid exchanges at nonanchor residues for the purpose of enhancing the interactions of the variant peptides with the TCR rather than MHC class I molecules. Nineteen variants of the wt $p53_{264-272}$ peptide were screened for their recognition by a bulk population of anti-wt $p53_{264-274}$-specific CTL that was maintained in our laboratory (7). T2 cells pulsed with the individual peptides at a fixed concentration of 1×10^{-6} M peptide served as targets for these CTL in a ^{51}Cr release cytotoxic assay. Significant cytotoxic reactivity against T2 cells pulsed with three of the 19 variant peptides, namely, 6T, 7W, and 7P, was detected (data not shown). Therefore, these three variant peptides were selected for further characterization.

Variant peptide binding to HLA-A2.1 molecules

Binding of the 6T, 7W, and 7P variant peptides to HLA-A2.1 molecules was compared with that of the parental peptide in an MHC stabilization assay. The relative mean fluorescence intensity of parental and variant peptide-stabilized HLA-A2 molecules on T2 cells is shown in Fig. 1. All the peptides showed stabilization of HLA-A2 molecules in a dose-dependent manner within the concentration range of 1×10^{-5}–1×10^{-9} M. However, in general, the binding affinities of the variant peptides to HLA-A2.1 molecules on T2 cells were slightly lower than that of the parental wt peptide (wt > 6T ≥ 7W > 7P).

Affinity of $p53_{264-272}$-specific CTL for variant peptides

The affinity of the bulk population of anti-$p53_{264-272}$-specific CTL for the variant peptides was determined in a 4-h ^{51}Cr release assay using T2 cells pulsed with these peptides at concentrations ranging from 1×10^{-5} to 1×10^{-12} M as target cells. As shown in Fig. 2, at concentrations $<1 \times 10^{-8}$ M, the dose-response curves of the three variant peptides were shifted to the left relative to that of the parental wt peptide. Because the increased responsiveness of the CTL for these variant peptides cannot be attributed to enhanced binding to HLA-A2.1 molecules, these results are consistent with an increased affinity of TCR for the variant peptides.

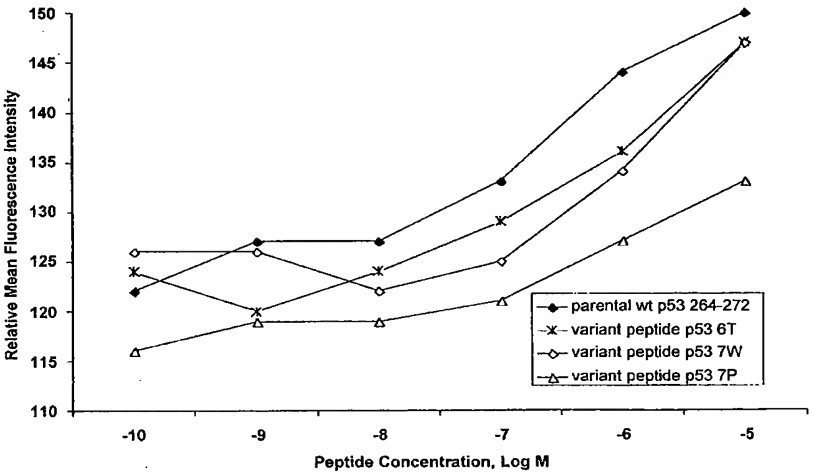

FIGURE 1. Identification of three HLA-A2.1-binding variant peptides of the wt $p53_{264-272}$ epitope. T2 cells were incubated with parental $p53_{264-272}$ peptide (LLGRNSFEV) or 6T, 7P, or 7W variant peptides at final concentrations of 1×10^{-5}–1×10^{-10} M. The relative mean fluorescence intensities of FITC-conjugated anti-MHC class I mAb (W6/32) are indicative of peptide-stabilized MHC class I molecules on T2 cells.

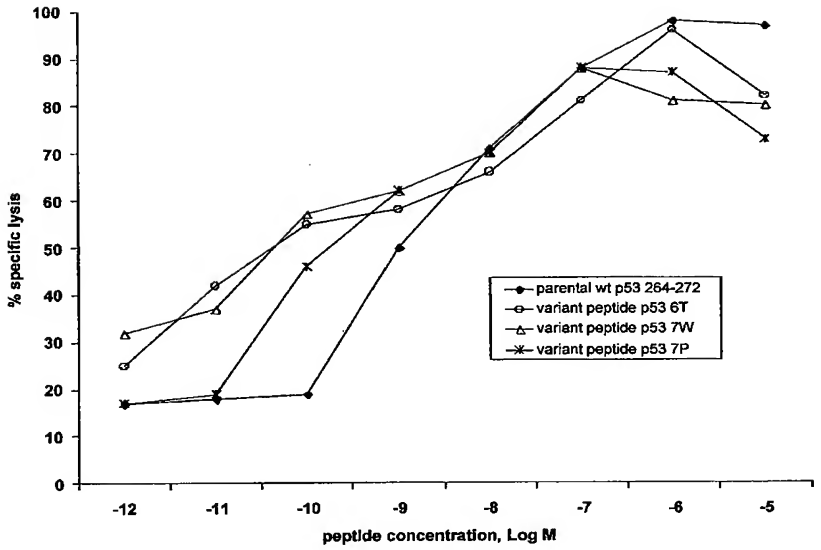

FIGURE 2. Variant peptides are recognized by anti-wt $p53_{264-272}$-specific CTL line. T2 cells were pulsed with different peptide concentrations and tested as targets in a 4-h ^{51}Cr release assay at the E:T ratio of 10:1.

Characterization of wt $p53_{264-272}$-specific CTL generated from PBMC obtained from normal donors using variant peptides

Previously, we reported that CTL reactive against the wt $p53_{264-272}$ epitope could be generated from PBMC obtained from only two of the seven HLA-A2.1$^+$ normal donors tested (12). Analyses involving multiple cryopreserved samples derived from leukopaks obtained from two of the normal donors (a responder and a nonresponder) confirmed the consistency of responses of these donors' PBMC to the parental peptide. In the same experiments in which the seven donors' PBMC were tested for induction using the parental peptide, CTL reactive against this peptide could be generated from five of these seven PBMC using either the 6T or 7W variant peptide. Included in this group were three nonresponsive PBMC (Table I); PBMC obtained from donors 6 and 7 responded to the 7W variant, while PBMC obtained from donor 4 responded to the 6T peptide. None of the seven PBMC tested responded to the 7P variant peptide.

The bulk populations of variant-induced cells generated from PBMC obtained from donors 6 and 7 effectively recognized and lysed T2 cells pulsed with the parental peptide in ELISPOT for IFN-γ and cytotoxicity assays. Fig. 3, *A* and *B*, shows the results obtained with the effectors generated from PBMC obtained from donor 7. Unpulsed T2 target cells or T2 cells pulsed with an irrelevant HLA-A2.1-binding peptide, the melanoma-associated gp100 peptide (33), were not recognized by these CTL in either assay to any noticeable extent. More importantly, these effector cells were also capable of recognizing the naturally presented epitope, as evidenced by their ability to lyse PCI-13 and SCC-9 tumor cells as well as mutant p53-transfected SaOS-2 Cl3 cell lines in a MHC class I-restricted manner (Fig. 3*C*). No significant cytotoxicity was noted against HLA-A2$^+$ tumor cell lines, SCC-4 and SaOS-2, which do not present the epitope.

The CTL generated from PBMC obtained from donor 4 using the 6T peptide yielded effectors with reactivity comparable with that of the 7W variant-induced CTL. The 6T-induced T cells were responsive to wt $p53_{264-272}$-pulsed T2 cells in the ELISPOT for IFN-γ assay (Fig. 4*A*), and cytolytic against the OSCC lines, SCC-9 and PCI-13, as well as SaOS-2Cl3 (Fig. 4*B*). This response was blocked by anti-HLA class I mAb but not anti-HLA-DR mAb. No significant reactivity was obtained against the tumor cell line SCC-4. The reactivity of these effectors against SaOS-2 cells in the analysis shown in Fig. 4*B* was higher than normally detected against this $p53^{null}$ cell line, using bulk populations of anti-p53 effectors (7, 12). However, the reactivity of the 6T-induced effectors against SaOS-2 targets was not significantly blocked by anti-HLA class I mAb and thus could be attributed to nonspecific effectors present in the bulk population. In summary, variant-induced effector T cells had similar reactivities against the parental epitope as those reported previously for the parental peptide-induced effectors from responsive normal donors as well as OSCC patients (7, 12).

Table I. *Summary of the anti-p53 CTL responses of PBMC obtained from normal donors following IVS using variant $p53_{264-272}$ peptides*[a]

Donor	Anti-p53 CTL Response After IVS With			
	wt $p53_{264-272}$	Variant 6T	Variant 7P	Variant 7W
1	+	−	−	+
2	+	−	−	+
3	−	−	−	−
4	−	+	−	−
5	−	−	−	−
6	−	−	−	+
7	−	−	−	+

[a] PBMC were stimulated with the peptide-pulsed autologous DC or PBMC in two to four IVS cycles. Effector cell reactivity was tested in ELISPOT and cytotoxicity assays; − indicates that no specific reactivity against the wt $p53_{264-272}$ peptide was observed, while + indicates that effectors were reactive against variant and parental peptides. Results using the parental wt $p53_{264-272}$ were reported in a previous publication from this laboratory (12).

Characterization of wt $p53_{264-272}$-specific CTL generated from PBMC of a nonresponsive OSCC patient using a variant peptide

The critical test of the variant peptides was whether their use could induce CTL capable of recognizing the anti-wt $p53_{264-272}$ epitope from nonresponsive patients whose tumors were considered capable of presenting this epitope (12). The nonresponsiveness of PBMC obtained from at least one of these donors, patient 3, has been repeatedly confirmed during the past 2 years using blood samples obtained at different times, as well as multiple cryopreserved leukapheresis samples obtained from this patient. As shown in Table II, none of the PBMC from three of these patients responded to the 6T or 7P variant peptides. However, the 7W variant

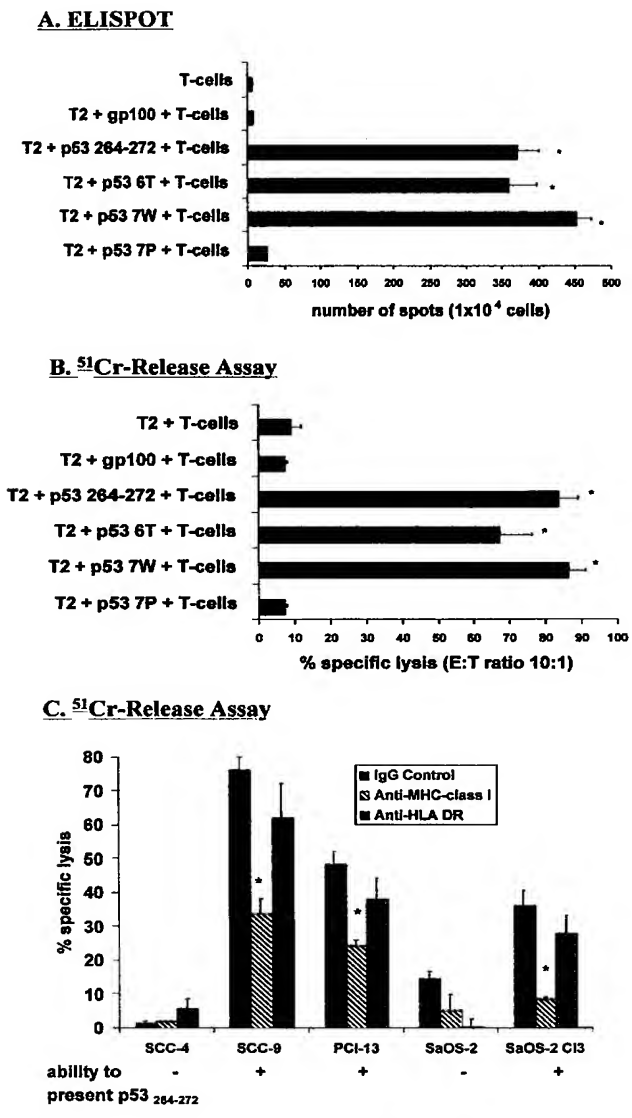

FIGURE 3. The 7W variant peptide-induced effectors induced from PBMC obtained from a nonresponsive healthy donor recognize the parental wt $p53_{264-272}$ peptide pulsed onto target cells or naturally presented by tumors. *A*, Recognition of peptide-pulsed T2 cells in ELISPOT for IFN-γ assays. Effectors were tested against T2 cells pulsed with an irrelevant gp100 peptide, the wt $p53_{264-272}$ peptide, or the variant peptides at 10 μg/ml. *B*, Lysis of T2 cells pulsed with various peptides at an E:T of 1:10. *C*, Lysis of tumor targets naturally presenting the epitope at an E:T ratio of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. *, A significant ($p < 0.05$) difference relative to IgG controls.

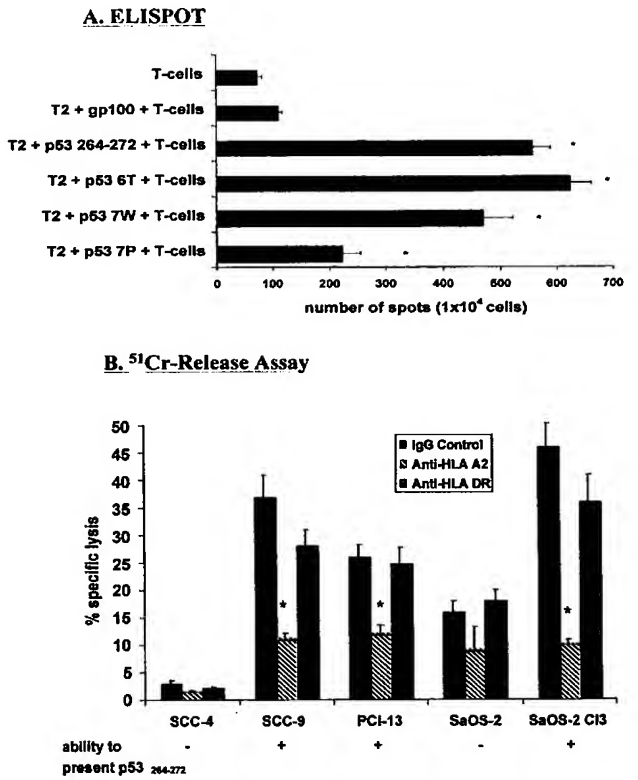

FIGURE 4. The 6T variant peptide-induced effectors induced from PBMC obtained from nonresponsive healthy donor recognize the parental wt $p53_{264-272}$ epitope pulsed onto target cells or naturally presented by tumors. *A*, Recognition of peptide-pulsed T2 cells in ELISPOT assays. Effectors were tested against T2 cells pulsed with either an irrelevant gp100 peptide, the wt $p53_{264-272}$ peptide, or a variant peptide at 10 μg/ml. *B*, Lysis of tumor targets naturally presenting the epitope at an E:T of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. *, A significant ($p < 0.05$) difference relative to IgG controls.

peptide did induce the ex vivo generation of anti-wt $p53_{264-272}$ CTL from PBMC of patient 3, whose autologous tumor, PCI-13, presents this epitope (7, 12). The affinity of these effectors for the parental epitope was comparable with that of a bulk population of CTL induced using the parental epitope, and was in the range of 1×10^{-9} M (Figs. 2 and 5*A*). Furthermore, the 7W-induced CTL were cytotoxic against a panel of tumor cell lines naturally presenting the wt $p53_{264-272}$ epitope, including the autologous PCI-13 cell line, and this reactivity was MHC class I restricted (Fig. 5*B*). This result clearly illustrates the potential value of the 7W variant peptide in immunotherapy targeting the wt $p53_{264-272}$ epitope in individuals like OSCC patient 3.

Tetramer-binding and TCR Vβ usage by T cell microcultures reactive against parental and/or variant peptides

The ability of the variant peptides to induce the generation of CTL specific for wt $p53_{264-272}$ from nonresponder PBMC raised the question of the relationship between these CTL and those induced by the parental peptide in responder PBMC. The need to investigate this relationship became evident when the cross-reactive bulk population of $CD8^+$ T cells induced with the 7W variant peptide from normal donor 7 was stained with the parental or variant tetramer. Whereas only ~2% $tetramer^{dim}$ cells were detected with the parental tetramer, a cluster of ~40% $tetramer^{bright}$ cells was detected with the 7W tetramer (Fig. 6, *A* and *B*). One possible explanation for this observed difference was that the variant peptide induced a single $CD8^+$ T cell population that bound the variant tetramer with higher avidity/stability than did the parental tetramer. Another possible explanation was that the variant peptide induced two distinct populations of $CD8^+$ T cells; one was cross-reactive and bound both tetramers (most likely with different avidities), while the other was specific for the 7W variant and bound the 7W tetramer with high avidity. The two possibilities could be

Table II. *Summary of the anti-p53 CTL responses of PBMC obtained from nonresponsive OSCC patients following IVS using variant $p53_{264-272}$ peptides*

Patient	Tumor[a]		Anti-p53 CTL Response After IVS[b]		
	p53 genotype	p53 protein	Variant 6T	Variant 7P	Variant 7W
1	Mutant R248W	+	−	−	−
2	Mutant V 157 F	+	−	−	−
3	Mutant E286K	+	−	−	+

[a] Patients' tumors were analyzed for genetic alterations in p53 exons 5–8, and the identified codon and missense mutations are denoted. The level of p53 expression in tumors was determined by immunohistochemistry, using anti-p53 mAb, and + denotes accumulation of p53 (12).

[b] PBMC were stimulated with the peptide-pulsed autologous DC or PBMC in two to four IVS cycles. Effector cell reactivity was tested in ELISPOT and cytotoxicity assays; − indicates that no specific reactivity against the wt $p53_{264-272}$ peptide was observed, while + indicates that effectors were reactive against variant and parental peptides.

distinguished based on TCR usage of the T cells involved in recognition of these peptides. To accomplish this, T cell microcultures were established by limiting dilution from bulk CTL populations induced with either parental or variant peptide. Several T cell clones from each type of microculture were expanded for further analysis. Based in part on their rates of proliferation as well as peptide specificities (Fig. 7), four oligoclonal T cell lines, designated 2, 4, 53, and 68, were selected for TCR analysis by complementarity-determining region (CDR)3 spectratyping.

Two of the cell lines analyzed, 53 and 68, were derived from the bulk population of 7W-induced CTL that was described above and shown to exhibit differential staining with the parental and 7W

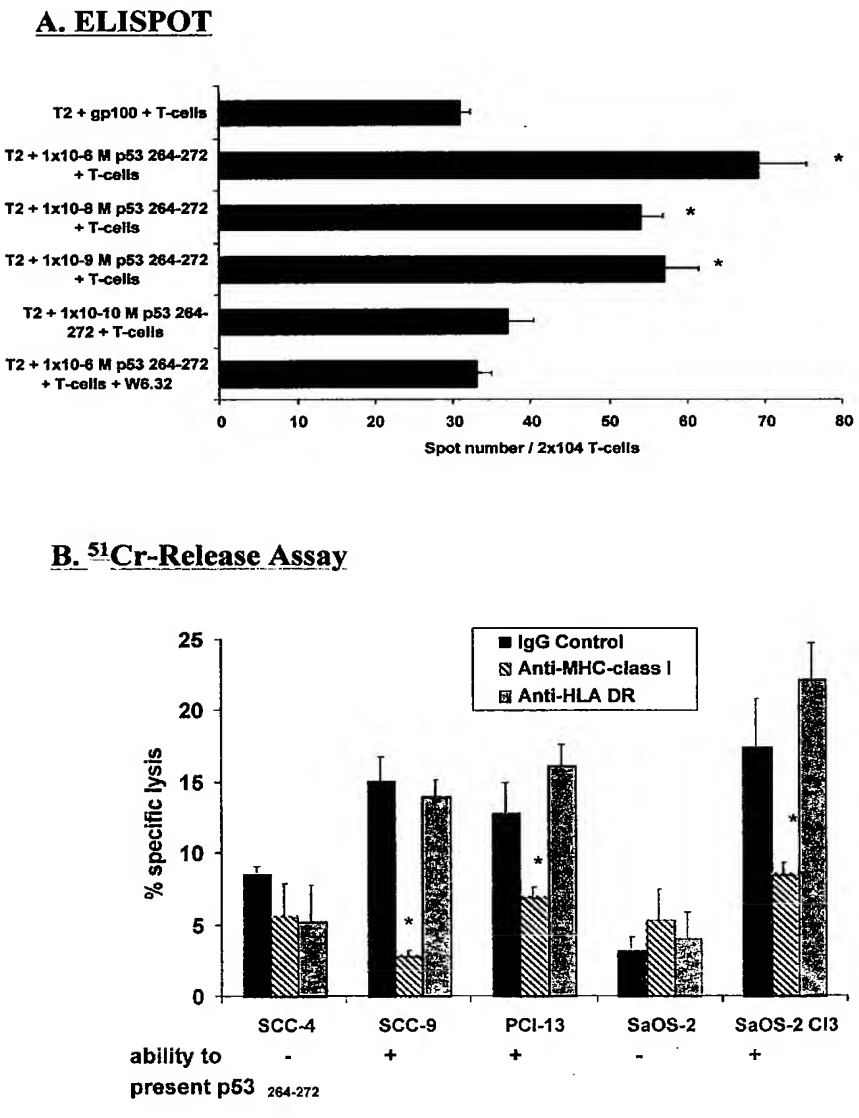

FIGURE 5. The 7W variant peptide-induced effectors obtained from a nonresponsive OSCC patient recognize the parental wt $p53_{264-272}$ peptide pulsed onto target cells or naturally presented by tumors, including PCI-13, the autologous tumor cell line. *A*, Affinity of effectors for the parental wt $p53_{264-272}$ peptide as determined in ELISPOT assay. T2 cells were incubated with parental peptide at concentrations of 1×10^{-6}–1×10^{-10} M. T2 cells pulsed with an irrelevant gp100 peptide served as a control. *B*, Lysis of tumor targets naturally presenting the epitope at an E:T of 40:1 in the presence or absence of anti-MHC class 1 or anti-HLA-DR mAb. *, A significant ($p < 0.05$) difference relative to IgG controls.

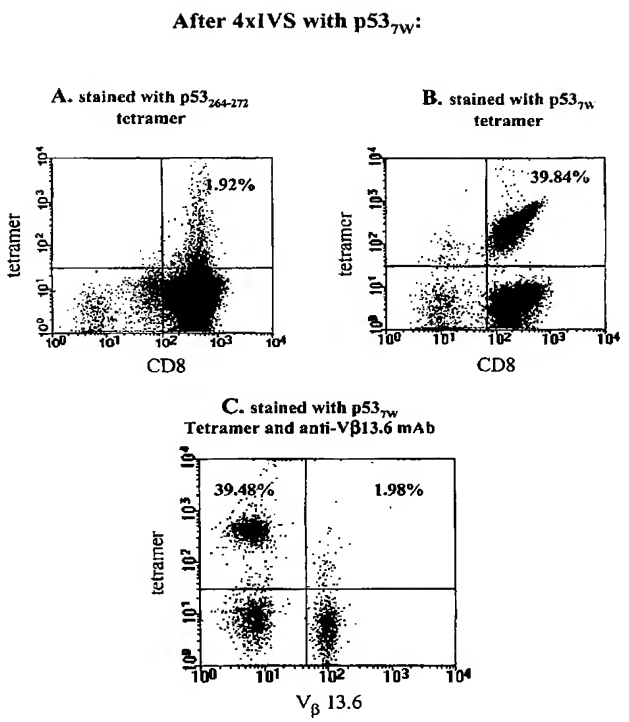

FIGURE 6. $CD8^+$ cells induced from PBMC of a nonresponsive normal donor using the 7W variant peptide that recognizes the parental peptide express TCR Vβ13.6. Three-color flow cytometry analysis of $CD8^+$ cells stained with HLA-A2.1 tetramers containing either the parental peptide (*A*), 7W variant peptide (*B*), or the 7W tetramer and anti-Vβ13.6 mAb (*C*). The numbers in the *upper right quadrants* indicate the percentage of $tetramer^+$ cells. The analyses shown in *A* and *B* involved 75,000 events, while in *C* 10,000 events were analyzed.

tetramers (Fig. 6). Although tetramer analysis of the bulk population suggested that the vast majority of CTL were 7W specific, one of the several T cell clones isolated was cross-reactive. The 53 cell line, which was specific for the 7W variant, was found to express Vβ9. The 68 cell line, which recognized the variant as well as the parental peptide, was found to express Vβ13.6 with completely different CDR3 and J regions from those expressed by line 53 (Table III). Consistent with these findings was the result of a combined tetramer/Vβ expression flow analysis of the bulk population from which the 53 and 68 cell lines were derived. We observed that the ~40% cells that stained as a distinct cluster with the 7W tetramer were $V\beta13.6^-$, whereas the ~2% cells that stained weakly with this tetramer were $V\beta13.6^+$ (Fig. 6*C*).

The other two cell lines analyzed, 2 and 4, were derived from bulk populations of effectors induced with the parental peptide from PBMC obtained from a patient (patient 2 in Ref. 12) and a normal donor (donor 2, Table I), respectively. The 2 cell line was found to express Vβ13.6, with identical motifs for the CDR3 and J regions as the 68 cell line, which was induced from a different individual using the 7W peptide (Table III). The observation that cross-reactive T cells induced by the variant peptide from PBMC obtained from one donor express the identical TCR/CDR3/J region as that expressed by T cells induced with the parental peptide from another donor illustrates the heteroclitic nature of the 7W variant peptide.

The 4 cell line was shown to express Vβ1 with a CDR3 and J region sequence distinct from those of any of the other cell lines analyzed (Table III). Interestingly, Vβ analysis of the bulk population of effectors from which the 4 cell line was isolated detected mainly Vβ1 and Vβ13 $CD8^+$ cells. Another bulk population of parental peptide-induced CTL, which was obtained from a different normal donor (7) and used to identify 7W and 6T peptides as potential APLs (Table II), was found to consist of >90% $V\beta1^+$ cells (data not shown). These results, summarized in Table IV, are strongly suggestive of a relatively limited TCR Vβ usage being involved in recognition by CTL of the HLA-A2.1-restricted, wt $p53_{264-272}$ epitope, regardless of whether these cells are induced by the parental or variant peptide.

Discussion

Most studies of APL of tumor Ags involve amino acid exchanges at anchor residue positions of the peptide to enhance its binding to class I MHC molecules. The recent modification of the HLA-A2.1-restricted wt $p53_{149-157}$ peptide at anchor position 2 to improve its binding to the restriction element and immunogenicity is one example of this approach (26). Particularly relevant to this study are variants designed to enhance TCR/peptide interactions rather than increase MHC binding, such as the HLA-A2.1-restricted, melanoma-associated MART1/Melan A_{27-35} and carcinoembryonic Ag, CAP1, peptides, which involve amino acid exchanges in residues other than anchor positions (18–21). While the binding affinities of these variant and parental peptides to HLA-A2.1 molecules are comparable, amino acid exchanges of these peptides at nonanchor positions yielded variant peptides that were more immunogenic than the parental peptides.

Since the parental wt $p53_{264-272}$ peptide has a reasonable affinity for HLA-A2.1 molecules ($>1 \times 10^{-9}$ M), the 19 $p53_{264-272}$ variants designed for this study had unmodified anchor positions. Among the amino acid exchanges tested, those at position 6 (6T) and position 7 (7W) appeared to be promising. Since both variants have lower affinities than the parental peptide for HLA-A2.1 molecules, their ability to increase the frequency of anti-$p53_{264-272}$ CTL responses generated from nonresponsive PBMC does not appear to be due to their enhanced binding to HLA-A2.1 molecules. Instead, their increased immunogenicity might be due to the replacement or counterbalancing of residues causing adverse TCR-peptide interactions. Such a replacement could result in an improved interaction of the peptide/MHC complex with TCR and a subsequent expansion of T cells capable of recognizing the parental epitope (23, 24). Two lines of evidence support this conclusion. First, using the parental tetramer to determine the frequency of $tetramer^+$ precursor T cells in unstimulated PBMC obtained from normal donors and patients with cancer, we found that most of the nonresponsive individuals had markedly lower frequencies of these cells in their peripheral circulation than did the responders (data not shown).[4] Second, the parental and variant peptides were found to engage and expand T cells expressing the same TCR in PBMC obtained from responsive and nonresponsive donors (see Tables III and IV). These findings support the concept that increased stability of interaction with the TCR is the basis for the enhanced functional activity of the 7W variant peptide.

Although the use of variant peptides did reverse the nonresponsiveness in IVS of PBMC obtained from some donors, their use did not yield high-affinity CTL. The persistence of low-affinity CTL against self tumor peptides, such as wt p53 epitopes, which is considered a true consequence of tolerance (15), might be due to a limited TCR repertoire being available for recognition of these epitopes. Our analyses detected the predominant use of only two TCR Vβ families, Vβ1 and Vβ13.6, being involved in CTL recognition of the wt $p53_{264-272}$ epitope in four different donors. Furthermore, in two different donors, identical usage by the parental

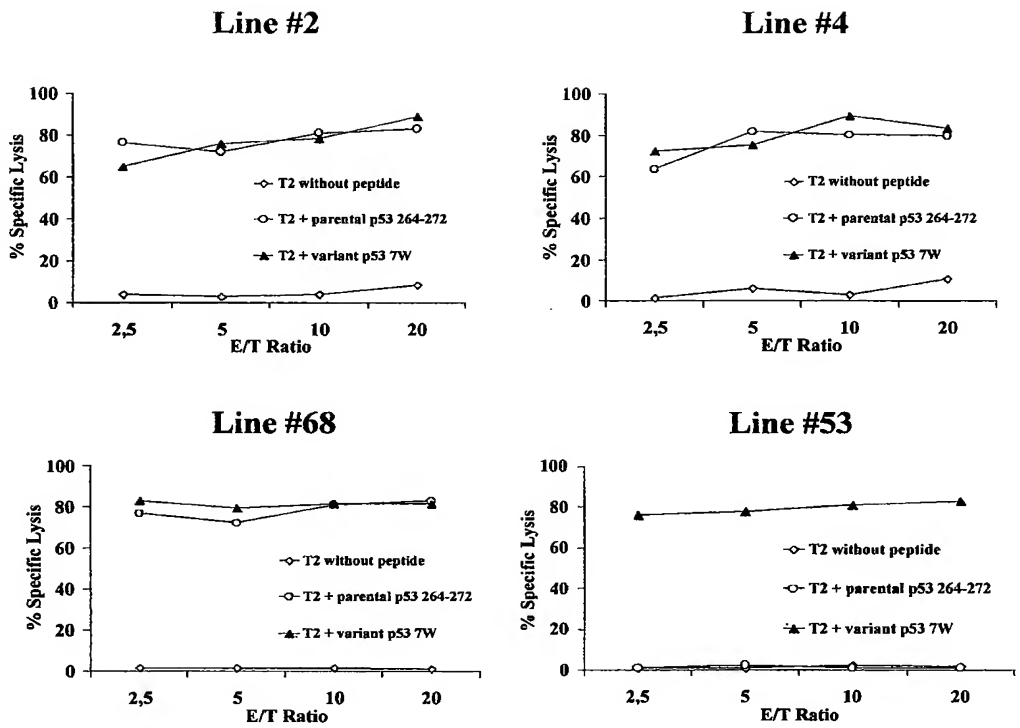

FIGURE 7. Specificity of T cell lines analyzed for TCR Vβ usage. Lines 2 and 4 were derived from bulk populations of effectors induced from an OSCC patient and a normal control, respectively, using the parental peptide. Both are cross-reactive against the variant peptide. Lines 53 and 68 were derived from a bulk population of effectors induced from a nonresponsive healthy donor with the 7W variant peptide. The 53 cell line is specific for the 7W variant, while the 68 cell line is cross-reactive.

and variant peptide was detected. In contrast, an analysis of responses in HLA-A2.1$^+$ patients to repeated immunizations with an anchor position-variant peptide of the melanoma-associated $gp100_{209-217}$ epitope demonstrated that the appearance of higher-affinity T cells was associated with an expansion of the TCR repertoire rather than an increased oligoclonal response (33). In the future, additional data on TCR usage of cross-reactive and variant-specific CTL cell could allow for extensive molecular modeling of the interactions within the trimeric complexes and, perhaps, the design of APL with more enhancing properties than those of the 6T and 7W variants. These variants might engage more diverse populations of T cells that are capable of cross-recognition of the parental epitope with, perhaps, higher avidity. However, the apparent outgrowth of epitope-loss tumors in OSCC patients responsive to this epitope suggests that even intermediate-affinity CTL recognizing wt $p53_{264-272}$ might be effective in tumor eradication (12).

To fully estimate the potential of p53-based vaccines in immunotherapy of cancer, it is becoming increasingly apparent that an array of T cell-defined wt p53 epitopes needs to be analyzed, and strategies for optimal induction of T cells recognizing these epitopes need to be further evaluated. In this regard, the use of genetically modified DC expressing intact wt p53 appears to enhance the generation and increase the frequency of antitumor effectors from PBMC of normal donors and cancer patients (34). The p53-based immunotherapy also might be critically dependent on targeting the right epitopes and matching a patient's ability to respond ex vivo to wt p53 epitopes with the potential of his/her tumor to present these epitopes for immune recognition. Again, of course, it is necessary to be aware that a patient's ex vivo responsiveness to these epitopes does not guarantee a successful in vivo response to immunization with them. In this study, HLA-A2.1$^+$ patient 3 with OSCC, for whom the tumor cell line and tumor-specific CTL are available in the laboratory, has been

Table III. *Amino acid sequences of monoclonal TCR transcripts expressed in four parental and/or variant $p53_{264-272}$-specific CTL lines*[a]

Line	IVS	Specificity	Vβ Family	Sequences		
				Vβ	CDR3 region	J region
2	wt	wt + 7W	Vβ13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3
4	wt	wt + 7W	Vβ1	LELGDSALYFCA	SSEGGL	ETQYFGPGTRL/BJ2-5
53	7W	7W	Vβ9	LGDSAVYFCA	SSAGTNT	YEQYFGPGTRLTVT/BJ2-7
68	7W	wt + 7W	Vβ13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3

[a] Lines 2 and 4 were stimulated with the parental wt $p53_{264-272}$ peptide (wt) and were reactive against the parental and 7W variant peptides. Lines 53 and 68 were stimulated with the 7W variant peptide. Line 53 was reactive against the 7W variant peptide only, while line 68 was reactive against the parental and 7W variant peptides. Single peaks in individual TCR variable chain families, suggesting clonality, were analyzed by direct sequencing of the PCR products.

Table IV. *Summary of evidence of limited TCR Vβ usage for CTL recognition of the wt $p53_{264-272}$ epitope*[a]

PBMC Donors[b]	Induced with $p53_{264-272}$ Peptide	Bulk T Cell Population Vβ Usage	Derived T Cell Lines[c]	
			Vβ usage	p53 peptide specificity
Normal donor 2 (R)	wt	Vβ1, 13.6	4 Vβ 1	wt/7W
Normal donor 4[d] (R)	wt	Vβ1	ND	
Normal donor 7 (NR)	7W	Vβ 9, 13.6	53 Vβ 9	7W only
			68 Vβ 13.6[e]	wt/7W
OSCC patient 2[f] (R)	wt	Vβ 13.6	2 Vβ 13.6[e]	wt/7W

[a] See Table III for the details on TCR Vβ usage.
[b] Normal donors and patients identified in Tables I and II; R, responsive to IVS of PBMC to the wt $p53_{264-272}$ peptide; NR, nonresponsiveness.
[c] T cell lines derived by limiting dilution.
[d] Normal donor used as source of PBMC for induction of a bulk population of CTL specific for wt $p53_{264-272}$. Generation and characterization of this cell line was detailed in Ref. 7.
[e] These T cell lines express identical Vβ, CDR3, and J region sequences (see Table III).
[f] The OSCC patient 2 identified in Table I in Ref. 12.

of particular interest. The tumor cell line established from this patient and designated PCI-13 accumulates p53 molecules expressing a missense mutation at codon 286 and naturally presents the $p53_{264-272}$ epitope, albeit following pretreatment with IFN-γ (7, 12). The ability to generate anti-$p53_{264-272}$ CTL with the 7W variant from this patient's PBMC, which were nonresponsive to the parental peptide, provides a basis for the potential use of the 7W variant peptide in immunotherapy of this patient and, perhaps, other nonresponsive OSCC patients with tumors expressing similar characteristics. Concurrently, it needs to be determined whether the trends observed in OSCC patients regarding their responsiveness to wt p53 epitopes and the potential of their tumors to present these epitopes are also apparent in patients with other types of cancers.

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ORIGINAL ARTICLE

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Functional characterization of CTL against gp100 altered peptide ligands

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Abstract In this study, four modified gp100 peptides were designed by combining amino acids from the melanoma peptide antigen $gp100_{(209\ 217)}$ with preferred primary and auxiliary HLA-A *0201 anchor residues previously identified from combinatorial peptide library screening with recombinant HLA-A*0201. These modified peptides demonstrated stronger binding affinity for the HLA-A*0201 molecule compared to wild-type gp100 peptide. Nine CTL lines generated from patients immunized with the g209-2 M peptide and one CTL line from a non-immunized patient were tested for the ability to respond to these modified gp100 peptides. Stimulation of CTL by two of four modified peptides induced higher levels of IFN-γ secretion than the wild-type gp100 peptide, demonstrating that higher peptide binding affinity for HLA molecules does not necessarily equate to functional activity of CTL. Two major and one minor CTL recognition pattern were observed, irrespective of previous peptide immunization, suggesting that multiple, rationally designed modified tumor peptides for the same epitope stimulate a broad CTL response by activating multiple CTL capable of cross-reacting with the natural antigenic peptide.

Keywords gp100 · Melanoma · CTL · HLA
Altered peptide ligands

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Introduction

gp100 is a melanocyte lineage-restricted glycoprotein that is expressed by most melanoma cells and is recognized by T lymphocytes in the context of major hisotcompatibility complex (MHC) molecules [1]. One immunodominant gp100 peptide, residues 209–217 (ITDQVPFSV), is an HLA-A*0201 epitope that has been previously demonstrated to be recognized by tumor-infiltrating lymphocytes (TIL) isolated from melanoma patients [2]. Initial studies showed that the $gp100_{(209\ 217)}$ peptide presented by HLA-A2 molecules could be modified to enhance binding to the HLA class I molecule [3]. Rosenberg et al. vaccinated metastatic melanoma patients with the modified $gp100_{(209\ 217)}$ peptide, g209–2M, in combination with interleukin-2 (IL-2) therapy. Peptide vaccination led to objective clinical responses in 38% of patients [4]. These clinical findings confirmed that epitope-specific CTL could be induced in cancer patients as a result of vaccination and this induction correlated with partial or complete tumor responses. These studies and others demonstrate the feasibility of designing modified tumor-specific antigenic peptides as vaccine therapeutics [5, 6, 7].

We previously determined preferential auxiliary anchor residues for the HLA-A*0201 molecule using a novel synthetic peptide bead library screening technique [8, 9]. The screening technique was performed by incubating recombinant HLA-A*0201 molecules in the presence of β_2m and random nonamer peptide beads. Using this approach we found that aromatic amino acids, especially phenylalanine (F), were most frequently present at the first and third positions. Leucine (L) and valine (V) were preferred at positions two and nine, respectively, agreeing with previous findings [10, 11]. Furthermore, we observed that glutamic acid (E) was frequently present at position four in peptides that bound the HLA-A*0201 molecule. Therefore, we attempted to increase the immunogenicity of the $gp100_{(209\ 217)}$ melanoma peptide by incorporating

preferred amino acid residues at positions that resulted in improved binding to the HLA-A*0201 molecule.

Using CTL lines generated from patients with melanoma who were immunized with g209–2M, we report the recognition profile of a panel of CTL lines with four HLA-A*0201-binding peptides derived from $gp100_{(209\ 217)}$ and correlate peptide affinities for the HLA-A*0201 molecule with the effects on CTL responses in vitro. The results presented here demonstrate that multiple altered peptide ligands that are antigenically cross-reactive with wild-type peptide are capable of stimulating existing CTL from cancer patients.

Materials and methods

HLA-A*0201-binding synthetic peptides

The HLA-A*0201-binding peptides used in this study were gp100 (residues 209–217, ITDQVPFSV) and its modified derivatives, g209–2M (IMDQVPFSV), APL 1 (FLFEVPFSV), APL 2 (FLDQVPFSV), APL 3 (FLDEVPFSV) and APL 4 (ILDQVPFSV). The HLA-A*0201-binding peptide GILGFVFTL (influenza virus matrix peptide, M1: 58–66) was used as a negative control. Hepatitis B virus core antigen (residues 18–27), an HLA-A*0201-binding peptide, (FLPSDFPSV) was synthesized with a cysteine residue substituted for the tyrosine residue (FLPSDCFPSV). This cysteine residue was conjugated to fluorescein (Fl-peptide) for use in competitive inhibition studies to measure the affinity of $gp100_{(209\ 217)}$ altered peptide ligands for the HLA-A*0201 molecule [12]. All peptides were synthesized by standard FMOC chemistry techniques, and soluble peptides were purified to >90% by high performance liquid chromatography and tested for purity by mass spectroscopy (a kind gift of Kit S. Lam, University of California, Davis, Sacramento, Calif.).

Cultured cell lines

All CTL lines were derived as previously described [13, 14]. The melanoma-specific CTL lines, which recognize a gp100-derived epitope g209–2M (IMDQVPSFV), were derived from fine-needle aspirates isolated from melanoma patients after immunization with the g209–2M peptide (with the exception of H.3–1, which was derived from a non-immunized melanoma patient [14]). CTL lines were cultured in Iscove's Modified Dulbecco's medium (IMDM; Life Technologies, Rockville, Md.) with 25.0 mM HEPES, supplemented with 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, Calif.), 1,000 U/ml penicillin-streptomycin, 2.0 mM L-glutamine and 0.5 mg/ml amphotericin B (ICN, Costa Mesa, Calif.). IL-2 (1,000 IU/ml; Chiron, Emeryville, Calif.) was added to cells every 2–3 days. T2 cells (CRL-1992, American Type Culture Collection, Manassas, Va.) were cultured in RPMI 1640 medium (Mediatech Cellgro, Herdon, Va.) supplemented with 10% heat inactivated fetal bovine serum (Omega Scientific, Tarzana, Calif.), 1,000 U/ml penicillin-streptomycin and 2.0 mM L-glutamine (cRPMI 1640 medium). Melanoma cell lines derived from fresh tumor isolates were established at the Arizona Cancer Center. J1 ($gp100^+$, $HLA\text{-}A2^+$), 2515 ($gp100^-$, $HLA\text{-}A2^+$) and H1 ($gp100^+$, $HLA\text{-}A2^-$) melanoma lines were cultured in cRPMI 1640 medium.

HLA-A*0201 binding affinity of gp100 altered peptide ligands

In order to measure the affinity of the APL for HLA-A*0201 molecules, competitive peptide inhibition assays were performed as previously described with slight modifications [12]. T2 cells (1×10^7 total cells) were washed twice with RPMI 1640 medium in the absence of serum (iRPMI). Recombinant β_2m (2.0 μg/ml) and reference peptide (Fl-peptide), (FLPSDCFPSV, fluoresceinated at the cysteine residue (C_6), 1.0 μg/ml), were added to T2 cells (2.5×10^5 cells/200 μl iRPMI/tube) and incubated for 16–18 h with varying concentrations of modified gp100 peptides at 26°C in a 5% CO_2 incubator. T2 cells were washed with PBS prior to flow cytometric analysis (FACScan flow cytometer, BD Biosciences, Mountain View, Calif.). Mean Fluorescence Intensity (MFI) values were used to determine inhibition of the Fl-peptide from binding to HLA-A*0201 molecules on T cells by modified gp100 peptides. Acquisition and analysis of data was performed using Cell Quest software, version 4.0 (Beckton Dickinson). Percent inhibition was calculated as: $[1\text{-}((MFI\ T2 + Fl\text{-}peptide + APL) - (MFI\ T2 + \beta_2m))/((MFI\ T2 + Fl\text{-}peptide) - (MFI\ T2 + \beta_2m))]\times100$. The IC_{50} of gp100 APL was determined by calculating the concentration of peptide required to inhibit binding of the Fl-peptide to T2 cells by 50% (IC_{50} in μg/ml). Trendlines were generated to determine IC_{50} values for each peptide.

Assessment of antigen recognition by CTL: IFN-gamma release assay

CTL were incubated with bead-bound peptides in cIMDM for 18 h at 37°C in 96-well round-bottom plates. Supernatants were harvested, and the concentration of IFN-γ was determined by ELISA using an OptEIA human IFN-γ set (Pharmingen, San Diego, Calif.) according to the manufacturer's instructions.

Measurement of CTL lytic activity by ^{51}Cr release

Chromium^{51} release cytotoxicity assays were performed to evaluate the ability of CTL to lyse the following melanoma cell lines: J1 ($A2^+$, $gp100^+$), 2515 ($A2^+$, $gp100^-$) and H1 ($A2^-$, $gp100^+$). Standard ^{51}Cr release methods were performed as previously described [15]. Target cells (5×10^3/well) were incubated with CTL at various E:T ratios for 8 h. Chromium^{51} released from target cells was measured using a Top Count microplate scintillation counter (Packard, Meriden, Conn.). Percent specific lysis was calculated as: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] ×100.

CTL-HLA-A2/peptide tetramer binding assay

HLA-A*0201-peptide tetramers (gp100, g209–2M, APL 2, 3 and 4) were generated by the NIAID Tetramer Core Facility. Tetramer staining was performed as previously described [16] with slight modifications. Briefly, H.3–1 CTL (1×10^5) were stained with phycoerythrin (PE)-conjugated HLA-A*0201/peptide tetramers (in PBS/5%FCS) for 1 h on ice followed by a 30 min incubation with PerCP-conjugated anti-CD8 mAb (Becton Dickinson, San Jose, Calif.). Cells were analyzed with a FACScan flow cytometer (BD Biosciences) using Cell Quest software, version 4.0 (Beckton Dickinson).

Results

Rational design of $gp100_{(209\ 217)}$ altered peptide ligands

Four modified gp100 peptides were designed by combining amino acids from the gp100 peptide with preferred HLA-A*0201 primary and secondary anchor residues (Table 1) previously identified by us from screening peptide libraries [8, 9] and by peptide elution of HLA-A*0201 molecules [10, 11]. gp100 APL were entered into an HLA-binding epitope prediction algo-

Table 1 Sequences, predicted HLA-A*0201 binding affinities and IC_{50} values of wild-type and modified $gp100_{(209\ 217)}$ peptides

	Sequence[a]	Epitope prediction[b]	IC_{50} (μg/ml)[c]
gp100	I T D Q V P F S V	18	23.03
g209–2M	I M D Q V P F S V	22	6.54
APL 1	F L F E V P F S V	26	51.16
APL 2	F L D Q V P F S V	23	3.25
APL 3	F L D E V P F S V	25	0.52
APL 4	I L D Q V P F S V	24	0.62

[a]Standard single letter aa code is used, and the residues shown in *bold* represent deviations from gp100 (209–217);
[b]peptide binding affinity for HLA molecules as predicted by the epitope prediction algorithm, SYFPEITHI [23];
[c]IC_{50} values determined by the amount of peptide needed to inhibit an HLA-A2 binding HBV reference peptide by 50%

rithm, SYFPEITHI [17], which scores peptides based on previously published HLA-A*0201-binding peptide motifs and preferred binding residues. Amino acids were assigned numerical values based upon anchor and auxiliary residues. All four peptides received higher HLA-A*0201-binding scores compared to the wild-type gp100 peptide (Table 1). These gp100 APL were synthesized on beads and tested for the ability to bind to the HLA-A*0201 molecule. In preliminary experiments, peptide beads were incubated with recombinant HLA-A*0201 molecules and β_2m and allowed to assemble into a tertiary complex. Peptide beads with stable HLA class I complexes were detected using a conformation-dependent mAb, W6/32, conjugated to alkaline phosphatase. All modified gp100 peptides bound to recombinant HLA-A*0201 as determined by a change in bead color, from colorless to turquoise (data not shown).

Modification of $gp100_{(209\ 217)}$ results in an increased affinity for the HLA-A*0201 molecule

he next objective was to measure the affinity of the gp100 APL for the HLA-A*0201 molecule using T2 cells. gp100 APL were tested for the ability to inhibit an HLA-A*0201-binding reference peptide (Fl-peptide), FLPSDCFPSV (HBV core $antigen_{(18\ 27)}$ peptide, fluoresceinated at the cysteine residue), from binding to HLA-A*0201 molecules. Relative peptide affinity for the HLA-A*0201 molecule was determined by incubating varying concentrations of APL with T2 cells, recombinant human β_2m and Fl-peptide (1.0 μg/ml) for 16–18 h at 26°C. Three peptides, APL 2, 3 and 4, demonstrated higher relative binding affinities for HLA-A*0201 molecules compared to wild-type gp100 (data not shown). The IC_{50} of the modified gp100 peptides was calculated by determining the amount of peptide needed to competitively inhibit the binding of the Fl-peptide to HLA-A*0201 molecules by 50% (Table 1). The native wild-type peptide $gp100_{(209\ 217)}$ had an IC_{50} of 23.03 μg/ml. APL 2, APL 3 and APL 4 bound with greater affinity compared to wild-type gp100 and therefore had lower IC_{50} values (3.25 μg/ml, 0.52 μg/ml and 0.62 μg/ml, respectively). APL 1 did not bind strongly to cell surface HLA-A2 molecules, as demonstrated by a two-fold higher IC_{50} value compared to the parental gp100 peptide.

Recognition of gp100 modified peptides by g209 2M-specific CTL

To determine whether the increase in relative affinity for HLA molecules observed with the modified gp100 peptides correlated with increased immunogenicity, the peptides were evaluated for the ability to stimulate IFN-γ production by CTL lines derived from patients with melanoma. We have previously reported that CTL are capable of presenting peptide to other CTL in the context of MHC molecules resulting in stimulation of the cells as measured by IFN-γ production [18]. Furthermore, IFN-γ secretion by CTL was comparable in the presence or absence of T2 antigen presenting cells [18]. Therefore, CTL were incubated with modified gp100 peptides for 18 h, and supernatants were harvested and assayed for IFN-γ by ELISA. CTL stimulation by gp100 altered peptide ligands resulted in three distinct recognition profiles (Fig. 1A–C). Four CTL lines, H.3–1, F1H5, F2C3 and K8B7, produced IFN-γ in response to two modified peptides, APL 2 and APL 4 (Fig. 1A). APL 2 has a phenylalanine (F) and a leucine (L) at positions 1 and 2, respectively, compared to isoleucine (I) and threonine (T) of the wild-type. In APL 4, a leucine (L) residue was substituted for a threonine (T) at position 2. The wild-type $gp100_{(209\ 217)}$ and g209–2M peptides also induced IFN-γ production from these CTL lines. APL 2 and APL 4 stimulated IFN-γ production that was comparable to stimulation by g209–2M. Incubation of CTL with an HLA-A2-binding influenza matrix peptide yielded no IFN-γ production.

Four CTL lines, K8E6, S2G12, F1B12 and W1C6, secreted IFN-γ in response to APL 4 (Fig. 1B). Again, IFN-γ secretion was observed when CTL lines were cultured with g209–2M; however, only the K8E6 CTL line was stimulated by gp100. Activation by a control influenza matrix peptide was not observed.

W3B1 was the only CTL line that was stimulated by APL 3 (Fig. 1C). This peptide, which differs from gp100 by three amino acids, has the highest affinity for the HLA-A*0201 molecule compared to the gp100 APL tested. APL 3 contains a phenylalanine (F) and leucine (L) that were substituted for isoluecine (I) and threonine (T) at positions 1 and 2, respectively. At position 4 a glutamine (Q) replaced a glutamic acid (E) residue. Interestingly, gp100 or g209–2M did not stimulate the W3B1 CTL line. Finally, APL 1, which differs the most from the wild-type gp100 (first four residues of the wild-type peptide were altered), and has the lowest relative affinity of each of the four peptides, did not stimulate IFN-γ production by any CTL line tested.

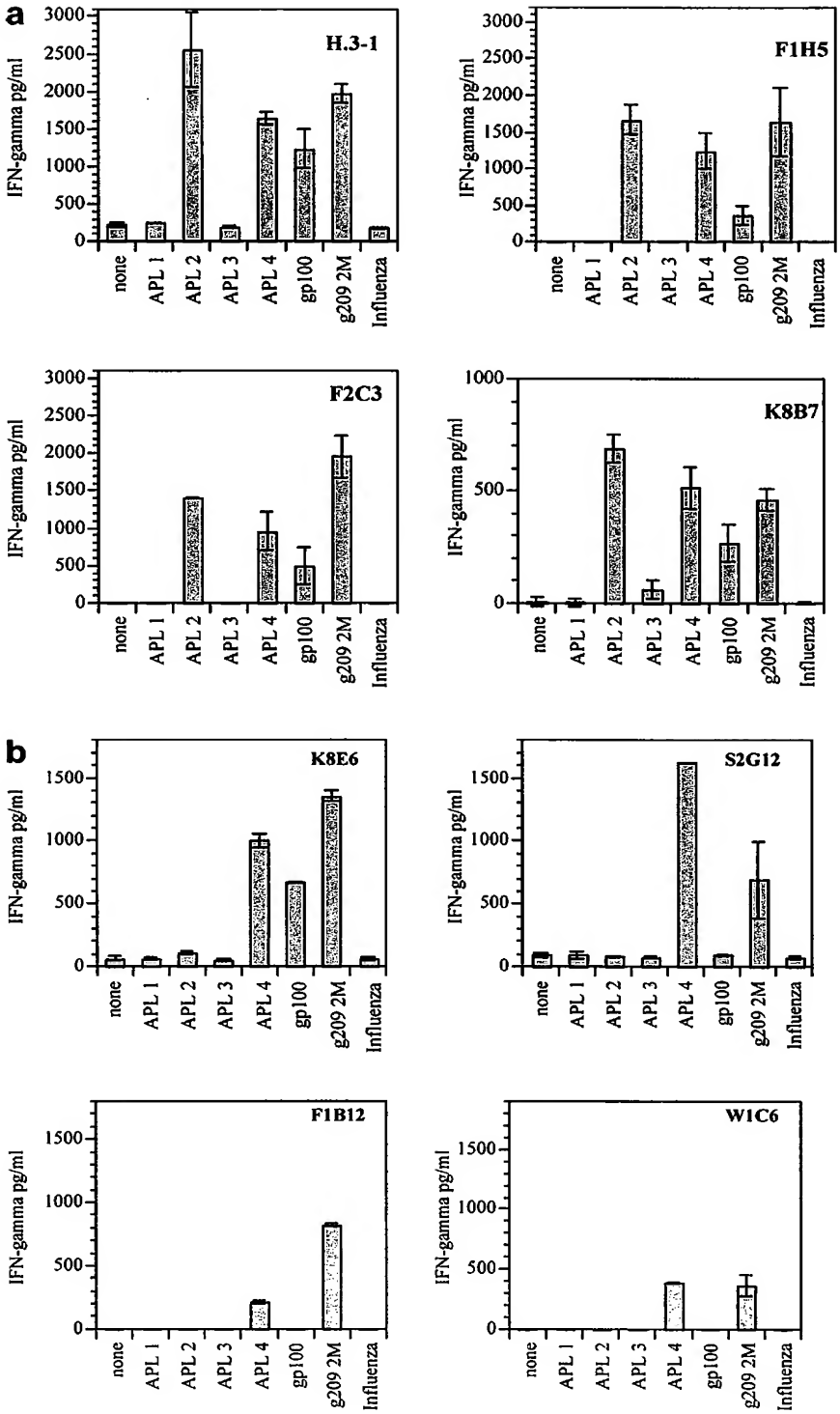

Fig. 1 IFN-γ production by CTL in response to gp100 peptides modified at HLA-A*0201-binding residues. CTL (5×10^3) were incubated with the indicated peptide beads for 18 h. Supernatants were harvested and assayed for the presence of IFN-γ

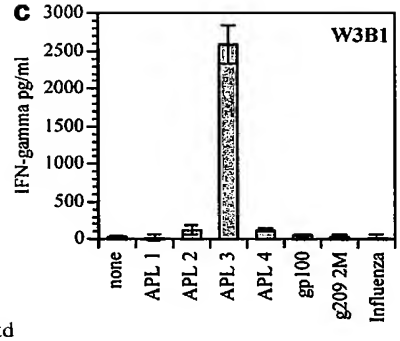

Fig. 1 Contd

H.3–1 CTL T cell receptor (TCR) demonstrates specific binding to HLA-A2/peptide tetramers

The H.3–1 CTL line, derived from a melanoma patient who was not immunized with g209–2M, was analyzed for T cell receptor (TCR) recognition of gp100 APL using HLA-A*0201/peptide tetramers. TCR/HLA binding assays revealed that H.3–1 bound strongly to gp100, g209–2M, APL 2 and APL 4, tetramers, but not APL 3, for which TCR binding was very weak (Fig. 2). APL 1 tetramers were not synthesized for tetramer binding assays, as this peptide did not stimulate IFN-γ production by any CTL lines tested. Mean fluorescence intensities (MFI) of H.3–1 tetramer binding illustrate that H.3–1 TCR bound to APL 2 and APL 4 with an MFI of 169 and 357, respectively, and demonstrated weak TCR binding to APL 3 (MFI 98). These results correlate with IFN-γ production that was observed (Fig. 1A). H.3–1 secreted IFN-γ in response to g209–2M, APL 2 and APL 4, while no IFN-γ secretion was detected in response to APL 3. As expected, a high MFI was observed for the TCR-HLA-A*0201/g209–2M tetramer interaction (MFI 829), since the H.3–1 CTL line was generated by stimulation with this modified gp100 peptide.

gp100 has a low affinity for the HLA-A*0201 molecule. While the H.3–1 CTL line secreted lower levels of IFN-γ when stimulated by wild-type gp100 compared to

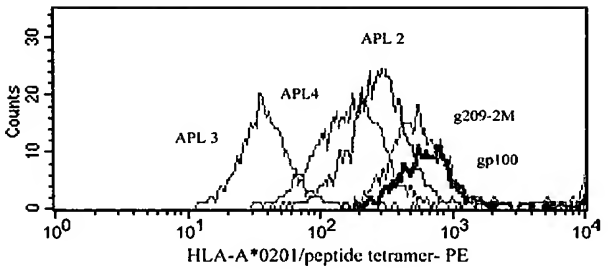

Fig. 2 Recognition of gp100 APL by H.3–1 CTL. The H.3–1 CTL line was incubated with the indicated PE-conjugated tetramer for 1 h on ice, followed by staining with PerCP-anti-CD8 antibody for 30 min. CTL were analyzed by flow cytometry and gated on $CD8^+$ cells prior to tetramer analysis

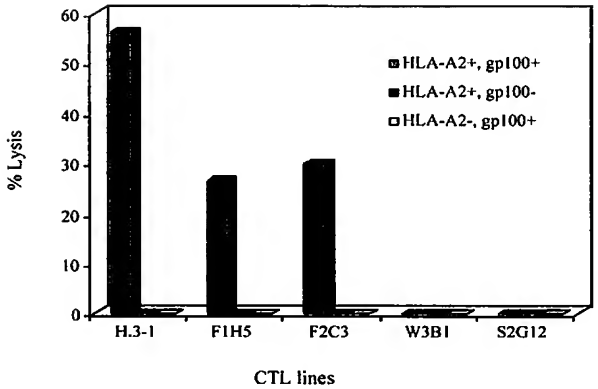

Fig. 3 Lysis of $HLA\text{-}A2^+$, $gp100^+$ melanoma cells by CTL lines that are stimulated by gp100 APL. CTL (2×10^5) were incubated with $chromium^{51}$-labeled melanoma cells (5×10^3 target/well). $Chromium^{51}$ release from target cells was measured after 8 h

gp100 APL (g209–2M, APL 2 and APL 4), tetramer analysis revealed that the H.3–1 TCR bound strongly to the HLA-A*0201/gp100 tetramer (MFI 627), further indicating that poor HLA peptide presentation results in poor CTL activation. HLA/peptide tetramer analysis also confirmed that the affinity of a peptide for the HLA molecule cannot predict TCR recognition or T cell activation by tumor-derived APL. For example, TCR recognition of gp100 APL 3 was the weakest compared to the other altered peptide ligands; however, APL 3 had the highest affinity for the HLA-A*0201 molecule.

Lysis of tumor cells expressing HLA-A*0201 and gp100 by CTL stimulated by gp100 APL

To determine whether CTL lines stimulated by gp100 APL could recognize native wild-type peptide, five CTL lines, selected on the basis of growth and availability, were tested for the ability to lyse gp100-expressing tumor cells. The H.3–1, F1H5 and F2C3 CTL lines lysed $HLA\text{-}A2^+$, $gp100^+$ target cells (Fig. 3), while $HLA\text{-}A2^-$, gp100+ and $HLA\text{-}A2^+$, $gp100^-$ melanoma cells were resistant to cytotoxicity. The W3B1 and S2G12 CTL lines did not lyse $HLA\text{-}A2^+$, $gp100^+$ tumor cells. These latter CTL lines did not secrete IFN-γ in response to wild-type gp100, suggesting that recognition of the parental peptide was lost. Therefore, these CTL would not be capable of lysing tumor cells expressing the gp100 protein. Importantly, three CTL lines derived from patients with metastatic melanoma were stimulated by gp100 APL and able to lyse $HLA\text{-}A2^+$, $gp100^+$ tumor cells.

Discussion

We report in this study several findings relevant to peptide-based cancer immunotherapy. First, we

demonstrated that custom-designed peptides with preferred HLA-A*0201 primary and secondary anchor residues with increased relative affinities for MHC molecules compared to wild-type peptide stimulate multiple different CTL lines from immunized and non-immunized melanoma patients. Second, we demonstrated that CTL lines derived from patients with melanoma are stimulated by these altered peptide ligands more strongly than wild-type gp100 peptide, as measured by IFN-γ production. Third, we confirmed that increased affinity for the HLA-A*0201 molecule does not necessarily correlate with increased peptide immunogenicity. Finally, we showed that CTL lines stimulated by altered peptide ligands are also able to recognize and kill naturally processed gp100 peptide presented by melanoma tumor cells.

A correlation exists between immunogenicity of peptides and HLA class I binding affinity [19]. As others and we have demonstrated, gp100 has a low binding affinity for HLA-A*0201 [3, 20]. Therefore modification or optimization of HLA class I-binding residues can result in peptides with higher affinity for HLA molecules and may result in increased immunogenicity [19, 21, 22, 23] (as observed with APL 2 and APL 4). However, designing peptides with higher affinities for HLA molecules does not always result in increased immunogenicity (APL 3). These results further demonstrate that an increased affinity for HLA class I molecules does not necessarily correlate with an ability to stimulate CTL.

Four out of nine CTL analyzed demonstrated APL 2 (FLDQVPFSV) recognition and secreted more IFN-γ compared to the wild-type peptide. APL 4 (ILDQVPFSV) stimulated eight CTL lines tested and these CTL secreted higher levels of IFN-γ in response to APL 4 compared to wild-type gp100 peptide. These findings are consistent with Parkhurst et al. [3], who reported that APL 2 and APL 4 (denoted 1F2L and 2L, respectively) had increased binding affinity for the HLA-A*0201 molecule and induced IFN-γ production from a TIL line specific for $gp100_{(209\ 217)}$. These data presented here suggest that multiple APL may be the best approach to stimulate diverse T-cell populations.

While the modifications incorporated into APL 3 (FLDEVPFSV) yielded an increased relative affinity for the HLA-A*0201 molecule, the majority of anti-gp100 CTL lines could not recognize this peptide. The amino acid substitutions at positions 1 and 2 in APL 3 were analogous to the changes made to APL 2. This suggests that while the introduction of a negatively charged amino acid at position 4 increases HLA class I affinity, conformational changes introduced into the tertiary structure of the MHC/peptide complex rendered it unrecognizable to anti-gp100 TCR. Therefore, CTL that recognize such MHC-peptide complexes would not cross-react with wild-type gp100 peptide presented by HLA molecules. Additionally, the amino acid residue at position 4 is highly likely to affect TCR binding, as it is adjacent to residue 5, an important TCR recognition residue. Thus, amino acid substitutions made in the center of the peptide may alter the positional arrangement of any or all residues within the peptide. Examination of substitutions made to other wild-type peptides demonstrated that subtle amino acid changes have significant effects on TCR contact residues [24, 25]. Therefore, while the amino acid modification introduced at position 4 contributed to increased affinity for the HLA-A2 molecule, it did not confer an increased stimulatory capacity for anti-gp100 CTL.

APL 1 (FLFEVPFSV) proved to have a significantly weaker relative affinity for HLA-A*0201 molecules than the parental gp100 peptide. Similar to the modifications introduced to APL 3, position 3 was also modified. Relative affinity studies indicate that the introduction of an aromatic residue (F) at position three, in addition to the three other amino acid substitutions, induced an entirely new peptide conformation in the HLA-A2 binding groove, affecting other amino acid residues within the peptide, resulting in poor binding to the HLA-A2 molecule and lack of CTL stimulation.

In summary, two out of four peptides designed based on both preferred HLA-A*0201 residues at position 1, and an anchor amino acid at position 2, are able to activate CTL lines generated from melanoma patients that retain gp100 recognition, as demonstrated by IFN-γ secretion. We have also demonstrated that modification of amino acids in positions 3 and 4 of the parental peptide result in a loss of peptide recognition by the TCR, further indicating that these residues may play a critical role in shaping the MHC-peptide structure necessary for T cell recognition.

Two CTL lines (W3B1 and S2G12) did not lyse gp100-expressing melanoma cells; albeit, these CTL lines were not activated by wild-type gp100 (209–217) peptide. IFN-γ was detected upon stimulation with APL 3 (W3B1), and g209–2M and APL 4 (S2G12). This suggests that in vivo, not all CTL activated by peptide immunotherapy would be capable of killing tumor cells. However, CTL activation by tumor-altered peptide ligands can result in cytokine secretion and aid in the recruitment of other immune cells that may contribute to tumor destruction.

While investigating the ability of gp100 APL to stimulate nine CTL lines generated from patients with metastatic melanoma it became evident that different lines responded to different peptides. These findings suggest that the administration of several APL for the same epitope may be more effective at stimulating a broader T-cell response in vivo. It is impossible to predict which group of patients will respond to certain tumor APL. A multi-peptide immunotherapuetic approach may be best to elicit an effective anti-tumor cellular immune response. In fact, in a clinical study by Banchereau et al. [6] in which patients with melanoma were vaccinated with autologous DC pulsed with multiple melanoma-derived peptides, regression of tumor metastases was observed in seven of ten patients who generated immu-

nity against more than two of the immunizing melanoma peptides. Hence, administration of multiple peptides for multiple epitopes may best serve to generate a potentially broadly applicable vaccination strategy for cancer patients.

Screening combinatorial peptide libraries with recombinant HLA class I alleles is an approach that others and we have employed to identify preferred HLA-binding residues [26, 27]. By combining the preferred residues with known motifs of tumor peptides, peptides with increased affinity for HLA molecules and potentially increased immunogenicity can be designed and synthesized. Our results demonstrate that peptides modified with preferred MHC-binding residues increase the affinity for HLA and may induce activation of CTL. However, mere prediction of peptide sequences that bind to MHC molecules with higher affinity need to be evaluated in vitro in order to determine if they induce effective CTL responses prior to use in vivo.

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ORIGINAL ARTICLE

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Her-2/*neu* altered peptide ligand–induced CTL responses: implications for peptides with increased HLA affinity and T-cell-receptor interaction

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Abstract In this study, we developed two Her-2/*neu*-derived E75 altered peptide ligands (APLs) that demonstrate increased affinities for the HLA-A*0201 allele compared with wild-type E75 peptide. The APLs contain amino acids from $E75_{(369\ 377)}$, an immunodominant Her-2/*neu*-derived peptide, and preferred primary and auxiliary HLA-A*0201 molecule anchor residues previously identified from combinatorial peptide library screening with the recombinant molecule. CTL lines were generated against wild-type E75 peptide (KIFGSLAFL) and APLs by multiple rounds of peptide stimulation of peripheral blood mononuclear cells (PBMCs) from HLA-$A2^+$ antigen normal individuals. CTL lines raised on wild-type E75 peptide cross-reacted with APLs and similarly, CTL lines raised on APLs cross-reacted with wild-type E75 peptide, as measured by IFN-γ ELISpot and target cell lysis assays. One of five individuals demonstrated specificity for APL 2 (FLFGSLAFL), whereas APL 5 (FLFESLAFL)-specific responses were observed from all five individuals tested. Molecular models of the E75, APL 2, and APL 5/HLA-A2 complexes indicated that the substitution of glycine with glutamic acid at position four of APL 5 resulted in the presentation of a large, negatively charged side chain that interacts with the outer edge of the HLA-A2 antigen alpha helix and is freely available to interact with cognate T-cell receptors. The results of this study further substantiate the concept that rational design of T-cell epitopes may lead to stronger peptide immunogens than natural, wild-type peptides.

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Keywords Her-2/*neu* · Altered peptide ligand (APL) · Cytotoxic T lymphocyte (CTL) · Human leukocyte antigen (HLA) · T-cell receptor (TCR)

Introduction

Twenty to forty percent of adenocarcinomas, including breast, ovary, pancreas, colon, and lung, overexpress the Her-2/*neu* protein. Her-2/*neu* overexpression occurs in both primary tumors as well as metastatic sites and expression is stable [1]. Overexpression is often associated with a poor prognosis, a metastatic phenotype, and resistance to chemotherapy [2, 3]. Fisk et al. identified E75 (KIFGSLAFL, residues 369–377) as an immunodominant peptide derived from Her-2/*neu* [4]. Four of four ovarian-specific CTL lines recognized the E75 peptide [4], and furthermore, other groups have also substantiated E75 as an important HLA-A2 antigen-restricted CTL epitope [5, 6]. Several clinical trials have demonstrated both the efficacy and safety of E75 peptide immunization of HLA-$A2^+$ antigen breast or ovarian cancer patients whose tumors overexpressed Her-2/*neu* [7, 8, 9, 10]. Collectively, these studies suggest that peptides derived from E75 may be potentially useful antigens in immunotherapies aimed at Her-2/*neu*-expressing tumors.

A current issue in the development of cancer vaccines is the induction of CTL responses against self-derived tumor antigens. Therefore, attempts have been made to overcome tolerance to self-antigens by increasing the immunogenicity of self-peptides. HLA-A*0201 allele-binding peptides are frequently nonamers, with positions two and nine considered primary anchor residues [11, 12]. These HLA anchor residues are essential, but alone are inadequate to bind to HLA class I alleles, indicating that other peptide residues are important for binding. Auxiliary anchor residues are considered critical for forming stable peptide-HLA class I complexes [13, 14, 15, 16]. Using a novel synthetic peptide bead

library screening technique, HLA-A*0201 allele secondary anchor residues were elucidated [17].

For peptide-based therapies to be effective, the HLA-peptide must be immunogenic. Several studies have shown that two components are important in determining the immunogenicity of HLA-peptide complexes: (1) the affinity of a peptide for MHC class I molecules and (2) the stability of the complex itself [18, 19, 20]. A direct correlation exists between immunogenicity of peptides and MHC class I binding affinity [18]. Peptides with high affinities for MHC class I molecules are present on cell surfaces for a longer time than peptides with low affinities for MHC class I molecules [21]. Therefore, peptides with increased affinity for MHC class I molecules have an increased potential to induce T-cell-mediated immune responses.

Modification of self-derived peptides may activate T cells by two potential mechanisms: (1) activation of existing T cells that are tolerant of, or have weak affinities to, self-antigens, or (2) activation of a new population of T cells specific for the modified tumor peptide that cross-react with wild-type peptide. The studies described here contribute to the rational design of effective peptide-based immunotherapies that aim to activate tumor-specific T cells.

Material and methods

HLA-A*0201 allele-binding synthetic peptides

The following HLA-A*0201 allele-binding peptides used in this study were Her-2/*neu* peptides: E75 (residues 369–377, KIFGSLAFL), modified E75 peptide 2 (APL 2, FLFGSLAFL), and modified E75 peptide 5 (APL 5, FLFESLAFL); and hepatitis B core antigen (FLPSDYFPSV, residues 18–27). The hepatitis B virus core antigen (FLPSDYFPSV) was synthesized with a cysteine residue substituted for the tyrosine residue (FLPSDCFPSV). This cysteine residue was conjugated to fluorescein (Fl-peptide) for use in competitive inhibition studies to measure the affinity of modified Her-2/*neu* E75 peptides for the HLA-A*0201 allele. Peptides were manufactured by PeptidoGenic Research (Livermore, CA) and were greater than 95% pure as assessed by HPLC and mass-spectrometric analysis.

Cell lines, media, and antibodies

T2 cells (CRL-1992; American Type Culture Collection, Manassas, VA) were used as antigen-presentation cells for ELISpot and ^{51}Cr release assays. T2 cells, K562 (NK-sensitive erythroblastoma cell line), and MCF-7 tumor cells (HLA-A2$^+$, Her-2/*neu*$^+$) were cultured in cRPMI 1640 medium (RPMI 1640 medium (Cellgro, Herdon, VA) supplemented with 10% heat-inactivated FBS (Omega Scientific, Tarzana, CA), 1,000 U/ml penicillin-streptomycin, and 2-mM L-glutamine). A Her-2/*neu*-overexpressing tumor cell line, MCF-7-2/18 (HLA-A2$^+$), was kindly provided by the Cancer Research Institute, University of California at San Francisco, and cultured in DMEM-G-418 (Dulbecco's Modified Eagle's Medium (Invitrogen, Rockville, MD) supplemented with 10% heat-inactivated FBS, 1,000 U/ml penicillin-streptomycin, 2-mM L-glutamine, and 500 μg/ml G-418).

A mouse antihuman HLA-A2 antibody (US Biologicals, Swampscott, MA) was used to identify HLA-A2$^+$ antigen normal individuals (1.0 μg/ml/1×10^6 cells) followed by staining of the HLA-A2-labeled PBMC with a FITC-conjugated goat antimouse antibody (1.0 μg/ml; BD Pharmingen, San Jose, CA). Acquisition and analysis of data was performed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA) and CellQuest software, version 4.0 (Beckton Dickinson).

HLA-A*0201 allele-binding affinity of modified Her2/*neu* E75 peptides

To measure the relative affinity of the APLs for HLA-A*0201 molecules, a competitive peptide inhibition assay was performed as previously described [22] with slight modifications [23]. Mean fluorescence intensity (MFI) values were used to determine inhibition of a reference peptide (Fl-peptide), FLPSDCFPSV, fluoresceinated at the cysteine residue (C_6) from binding to HLA-A*0201 molecules on T cells by modified E75 peptides. Percentage inhibition was calculated as (1 – [MFI T2 + Fl-peptide + modified peptide – MFI T2 only] / [MFI T2 + Fl-peptide – MFI T2 only]) x 100. The IC_{50} of the APL was determined by calculating the concentration of peptide required to inhibit binding of 50% of the Fl-peptide binding to T2 cells (IC_{50} in μM).

Generation of CTLs specific for Her-2/*neu* E75 and E75 APLs

PBMCs were obtained from normal donors either by leukapheresis (HSC-0096) or peripheral blood draws (HSC A01.88). Human blood was obtained from normal donors according to the guidelines set forth by the Human Subjects Committee at the University of Arizona. PBMCs were purified using standard white blood cell separation by density centrifugation with Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Piscataway, NJ). HLA-A2$^+$ antigen peptide-specific CTLs were generated as follows: on day 0, HLA-A2$^+$ PBMCs (2×10^6/ml) were plated in 2 ml of Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, Rockville, MD) with 25-mM HEPES, supplemented with 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA), 1,000 U/ml penicillin-streptomycin, 2-mM L-glutamine, and 0.5 mg/ml amphotericin B in 24-well plates. IL-7 (R&D Systems, Minneapolis, MN) was added at a concentration of 10.0 ng/ml to the cells. E75, APL 2, or APL 5 was added at 1.0 μg/ml. Starting on day 1, 300 IU/ml of IL-2 (Chiron, Emeryville, CA) was added every 2–3 days. Weekly peptide stimulations began 7 days after the first stimulation. Autologous, irradiated PBMCs were pulsed with 1.0 μg/ml peptide. Induction cultures (nonadherent lymphocytes) were transferred to wells containing peptide-pulsed irradiated cells in cIMDM (10:1 ratio of effector cells to stimulator cells). IL-2 was added every 2 to 3 days. At the end of the third stimulation, IFN-γ ELISpot assays were performed to determine peptide recognition.

Detection of IFN-γ-producing cells by ELISpot

Nitrocellulose plates (Millititer, Millipore, Bedford, MA) were coated with a mouse antihuman IFN-γ monoclonal antibody (10 μg/ml/well; Pharmingen, San Diego, CA), diluted in PBS overnight at 4°C. T2 cells (2.5×10^4 cells/well) were loaded with the indicated peptide (10.0 μg/ml) and incubated with effector cells (5×10^4 cells/well) in a total of 200.0 μl of X-Vivo 15 (BioWhittaker, Walkersville, MD). After incubation for 36 h at 37°C in 5% CO_2, cells were removed by washing five times with PBS / 0.05% Tween 20 (PBS-Tw). Captured cytokine was detected by incubation for 5 h at room temperature with biotinylated mouse antihuman IFN-γ monoclonal antibody (2.5 μg/ml/well, Pharmingen) diluted in PBS / 0.05% Tween 20 / 0.1% FCS (PBS-Tw-FCS). Streptavidin-HRP, diluted 1:1000 in PBS-Tw-FCS, was added for 1 h at room temperature. Staining was performed with 3-amino-9-ethylcarbazole (20.0 mg/ml, Sigma, St. Louis, MO) for 15–20 min at room temperature. Spots were counted by two individuals without well-identifiers, using a dissecting microscope.

Cytolytic activity of PBMCs cultured with Her-2/*neu* APLs

Chromium^{51} release cytotoxicity assays were performed to evaluate the ability of PBMCs to lyse the following target cells: T2 cells pulsed with 10.0 μg/ml of either wild-type E75 or HBV peptide, MCF-7, MCF-7-2/18, and K562 cell lines. Targets (5×10^3 cells/well) were labeled with 100 μCi of ^{51}Cr (Amersham Pharmacia Biotech, Piscataway, NJ) for 45 min followed by incubation with peptide (if indicated) in X-Vivo for 1 h prior to assay. Percentage specific lysis was calculated as ([experimental ^{51}Cr release − spontaneous release] / [maximum release − spontaneous release]) x 100.

Molecular modeling of the HLA-A2 molecule and E75/APL2/APL 5 complex

The coordinates of the HLA-A2 molecule complexed with an HIV-derived nonamer peptide, Tax, were downloaded from the Protein Data Bank (ID number 1DUZ) [24]. This file was used for manipulations in the Swiss-Pdb Viewer (DeepView) Model program [25]. The Tax peptide, which closely resembles the wild-type E75 peptide, was mutated manually to generate the E75, APL 2, and APL 5 peptides bound to the quarternary HLA-A2 antigen structure. Each HLA/peptide complex was submitted for energy minimization with the GROMOS96 feature of the Swiss-Pdb Viewer. Complexes were visualized in stereo using the computer graphics program *O* on a Silicon Graphics Octane computer to view all possible rotamers.

Statistical analyses

Lysis of target cells was compared using two-tailed, paired Student *t*-tests. Data sets are shown as mean ± SEM. Probability values of $p < 0.05$ were considered to indicate significant differences between data sets.

Results

Modified E75 peptides and HLA-A*0201 allele-binding affinity

Published HLA-A2 antigen-binding motifs suggest that the HLA-A*0201 allele-binding Her-2/*neu*-derived peptide, E75, does not contain optimal HLA-A2 antigen-binding residues and is unlikely to be presented as efficiently to CTLs as peptides with optimal or preferred amino acids for the HLA-A*0201 allele. Two E75 altered peptide ligands (APLs) containing optimal HLA-A*0201 allele-binding amino acids for the E75 peptide were designed for these studies (Table 1). Wild-type E75 contains a lysine (Lys) and isoleucine (Iso) at positions one and two, respectively. According to the HLA-A2 antigen motif previously reported by Smith et al. [17], phenylalanine (Phe) was found to be a preferred auxiliary residue at positions one and three, and leucine (Leu) was a preferred anchor residue (position 2) for nonamer peptides that bind to the HLA-A*0201 molecule. Therefore, these alterations were incorporated to the wild-type peptide for both Her-2/*neu* APLs (designated APL 2 and APL 5). The glycine (Gly^4) residue present at position four of E75 was replaced with a glutamic acid (Glu^4) in APL 5, again a preferred HLA-A*0201 allele-binding amino acid.

Table 1 Sequences, predicted HLA-A*0201 allele binding affinities, and IC_{50} values of wild-type and modified E75 peptides. Her-2/*neu* E75 was modified at HLA-A*0201 allele-binding residues. The affinity of these altered peptide ligands for the HLA-A*0201 allele was investigated using BIMAS (Bioinformatics and Molecular Analysis Section) that predicts half-time dissociation for HLA molecules based on coefficient tables

	Sequence[a]	BIMAS[b]	IC_{50} μM[c]
E75	K I F G S L A F L	481.19	27.0
APL 2	**F L** F G S L A F L	4,599.39	19.0
APL 5	**F L** F **E** S L A F L	18,857.49	15.0

[a]Standard single-letter amino acid code is used and the residues shown in ***bold*** represent deviations from Her-2/*neu* E75 (269–277)
[b]HLA half-time dissociation values calculated using BIMAS [14]
[c]IC_{50} values determined by the amount of peptide needed to inhibit an HLA-A2 allele-binding HBV reference peptide by 50%

To determine if the amino acid substitutions we introduced into the E75 peptide affected binding to the HLA-A2 molecules, we evaluated the APLs by computer predictions, and in vitro live cell binding assays (Table 1). The predicted half-time dissociation of the APLs for the HLA-A2 molecule was calculated using the BIMAS peptide-binding algorithm [26]. Based on coefficient tables, E75 APLs were assigned higher values compared with wild-type E75 (Table 1). To assess the relative HLA-binding affinity of E75 APLs, we employed a live cell binding assay to determine the amount of wild-type E75 and APL required to competitively inhibit the binding of an HLA-A2-binding fluoresceinated reference peptide (Fl-peptide) to the HLA-A*0201 allele, and calculated the concentration at which the Fl-peptide was inhibited by 50% (IC_{50}). The substitution of preferred anchor and auxiliary HLA-A*0201 allele-binding residues in place of non-optimal amino acids resulted in peptides with increased binding affinity for HLA-A2 molecules. Both APL 2 and APL 5 inhibited the binding of the Fl-peptide more strongly than wild-type E75 (Table 1). APL 5 demonstrated the highest relative affinity for the HLA-A*0201 allele compared with APL 2 and E75, and as predicted, APL 2 and APL 5 had lower IC_{50} values (19.0 μM and 15.0 μM, respectively) than wild-type E75 (27.0 μM).

Responses from CTLs raised on E75

We tested the immunogenicity of the APLs in normal HLA-A2$^+$ individuals. To address the capacity to prime CTL responses in vitro, PBMCs were stimulated with wild-type E75, APL 2, or APL 5, as described in "Materials and methods." PBMC cultures were monitored for peptide specificity by IFN-γ ELISpot assays after several rounds of peptide stimulation. Her-2/*neu* peptide-stimulated PBMCs with T2 cells pulsed with peptide (E75, APL 2, APL 5, or an irrelevant HLA-A*0201 allele-binding peptide) were cultured in IFN-γ ELISpot assays.

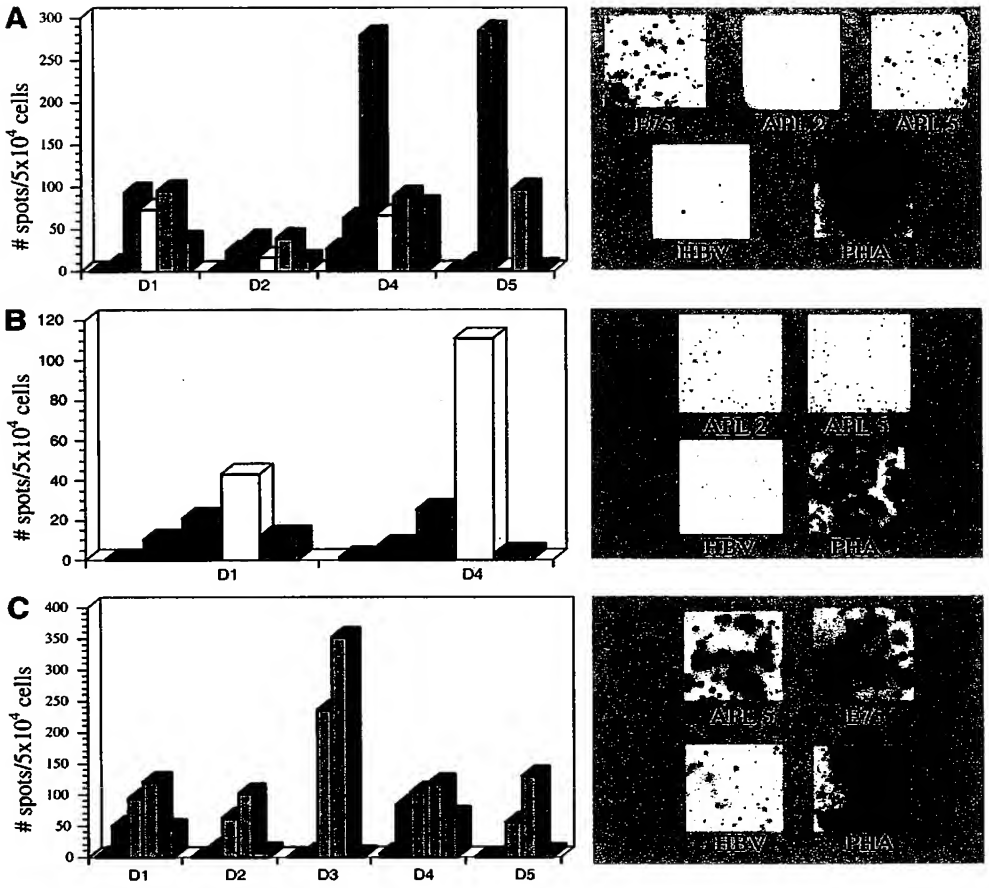

Fig. 1A–C Her-2/*neu* E75 and APL-specific responses detected from HLA-A2$^+$ antigen normal donors by IFN-γ ELISpot assays and representative wells of IFN-γ ELISpot. T2 cells (2.5×10^4 cells/well) pulsed with peptides (10.0 μg/ml) were used to stimulate bulk PBMC cultures (5×10^4 cells/well). T2 cells were pulsed with the following peptides: E75 *(green)*, APL 2 *(yellow)*, APL 5 *(pink)*, HBV *(purple)*, unpulsed T2 cells *(dark green)*, or CTL alone *(blue)*. **A** CTLs raised on E75 (results from donor 3 were not included, as no IFN-γ-secreting, peptide-specific CTLs were detected). Representative *spots* from donor 5 shown to the right. **B** CTLs raised on APL 2 (representative *spots* from donor 4 shown to the right). **C** CTLs raised on APL 5 (representative *spots* from donor 1 shown to the right)

Wild-type E75 peptide elicited E75-specific responses from CTL lines derived from three of five individuals tested (D1, D4, and D5; Fig. 1A). These wild-type E75-specific CTLs cross-reacted with Her-2/*neu* APL 5 (Fig. 1A). Transient E75-specific activity was detected from one donor and APL 5 cross-reactivity was also observed (D2; Fig. 1A). In general, IFN-γ secretion was not observed when E75-specific CTLs were challenged with APL 2.

Responses from CTLs raised on APL 2

Stimulation of PBMCs with APL 2 resulted in APL 2–specific responses from only one of five donors (D4; Fig. 1B). APL 2–specific CTLs demonstrated cross-reactivity to wild-type E75 (Fig. 1B). Transient IFN-γ production by PBMCs was observed from one donor when challenged with target cells pulsed with APL 2 (D1; Fig. 1B).

Responses from CTLs raised on APL 5

In contrast to APL 2, APL 5 consistently elicited CTL responses from all five individuals (Fig. 1C), suggesting that this peptide is more immunogenic than both wild-type E75 and APL 2. Of importance, CTL lines specific for APL 5 were stimulated by wild-type E75 (Fig. 1C).

Molecular modeling of the HLA-A2 molecule and E75 APL complexes

Both APL 2 and APL 5 bound with greater affinity to the HLA-A2 molecule compared with wild-type E75, but only the latter peptide proved to be immunogenic in five of five donors. Since our APLs only differed by one amino acid (APL 2 Gly^4, APL 5 Glu^4), we wanted to further understand the relationship of these peptides presented by the HLA-A2 molecule to the TCR to investigate if these mutations create different surfaces for TCR binding. Crystallographic information for the HLA-A2 molecule with bound peptide was available in the Protein Data Bank. The HIV-derived peptide, Tax, displays structural similarity to the Her-2/*neu*-derived E75 peptide. Therefore, we modeled the HLA-A2-E75 complex by replacing amino acids in Tax (LLFGYPVYV) with those of E75 (similar to that reported by [27]) using the Swiss Pdb Viewer Model program. CTL responses directed against APL 2 (**F**LFGSLAFL) were

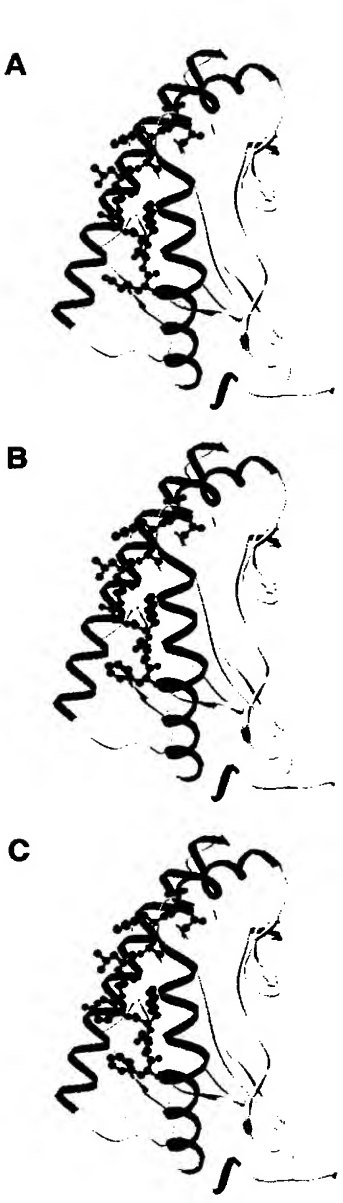

Fig. 2A–C Molecular modeling of the HLA-A2 molecule complexed with E75 altered peptide ligands. The HLA-A2 antigen alpha helices are displayed in *red* (*left:* α1 domain, *right:* α2 domain) and the beta sheet in *yellow*. **A** HLA-A2-E75. **B** HLA-A2-APL 2; amino acids different from wild-type E75 are denoted by a change from *blue* to *green*. **C** HLA-A2-APL 5; Glu^4 is represented by the *black residue*. The same orientation was used for all HLA-A2/peptide complexes

only detected from one of five individuals, whereas APL 5 (FLFESLAFL) stimulated specific responses from all donors tested. The glutamic acid residue introduced at position four in APL 5 resulted in a side chain that energetically points out of the binding pocket of the HLA-A2 molecule (Fig. 2, parts A, B, and C show E75, APL 2, and APL 5, respectively). The negatively charged Glu^4 residue in APL 5 (*black residue* in Fig. 2C) sits near positively charged amino acids, on the top edge of the α1 domain of the HLA-A2 antigen alpha helix (Arg^{65} and Lys^{66}), which are highly likely to play a role in peptide binding. Glutamic acid is a large residue compared with glycine and the carboxylate group may be better able to interact with the TCR, as the side chain points outward from the HLA molecule. Since the Glu^4 residue is the sole difference between the APL 5 and APL 2, the position of the side chain suggests that this residue plays a key role in the immunogenicity of the peptide, resulting in CTL activation in five of five individuals.

Cytolytic activity of APL-specific CTLs

To address whether IFN-γ-producing CTL lines could lyse targets cells pulsed with wild-type E75, cytotoxicity assays were performed. E75-specific CTLs lysed T2 cells pulsed with E75 peptide (Fig. 3A). Importantly, APL 2–specific and APL 5–specific cells lysed cells pulsed with wild-type E75 (Fig. 3, parts B and C, respectively). APL 5–specific CTLs showed increased lysis of wild-type E75-pulsed cells compared with APL 2-specific CTLs, correlating the results of our peptide affinity measurements and IFN-γ ELISpot assays. Collectively, CTLs from four of five individuals that demonstrated APL 5 specificity measured by ELISpot assays, lysed E75-displaying targets (D1, D3, D4, and D5; data not shown). APL 2 performed least well, inducing a specific response from one of five individuals as demonstrated by both IFN-γ ELISpot and cytotoxicity assays (D4). Although recognition of wild-type peptide by CTLs specific for both APL 2 and APL 5 occurred, lysis of MCF-7 (HLA-$A2^+$ and Her-2/*neu*$^+$) tumor cells was not observed. A tumor cell line transfected with a vector encoding the Her-2/*neu* antigen, MCF-7-2/18, was used to test CTLs specific for APL 5 (D1). Flow cytometric analysis demonstrated that these tumor cells overexpress Her-2/*neu*, compared with MCF-7 cells and express comparable levels of the HLA-A2 antigen (data not shown). Lysis of MCF-7-2/18 cells was observed, albeit low (7.39% ± 0.02%, the percentage lysis of the control, K562, was subtracted), at a 50:1 E:T ratio. When MCF-7-2/18 cells were pulsed with E75 peptide (10.0 μg/ml) prior to the cytotoxicity assay, a slight increase in lysis was observed (8.55% ± 0.001%).

Discussion

This study characterized two HLA-A*0201 allele-restricted, modified Her-2/*neu* E75 peptides for binding affinity to the HLA-A*0201 allele and for the ability to generate APL-specific CTLs that cross-react with wild-type E75 peptide. Our findings indicate that Her-2/*neu* APLs, designed for increased affinity for the HLA-A*0201 allele, can elicit specific peptide responses from normal donors. APL 5 had a significantly increased

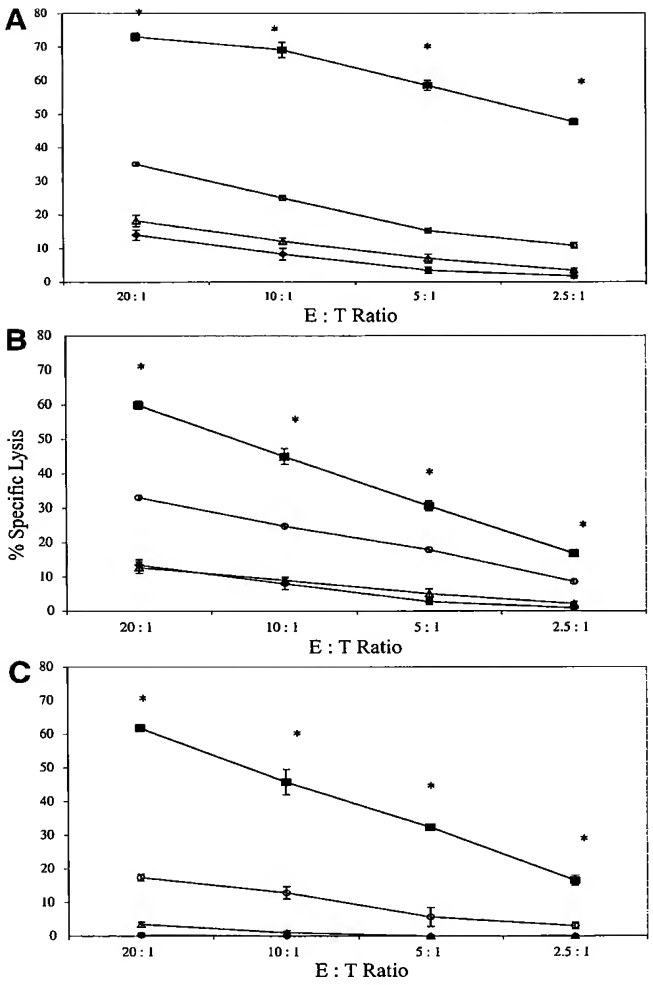

Fig. 3A–C Lysis of target cells pulsed with wild-type E75 peptide. CTLs (at the indicated E:T ratios) were incubated with ^{51}Cr-labeled target cells (5×10^3 cells/well), and ^{51}Cr release from target cells was measured after 6–8 h. **A** E75-specific CTLs, **B** APL 2–specific CTLs, and **C** APL 5–specific CTLs lyse T2 cells pulsed with E75 *(solid square)*. Lysis of the NK-sensitive cell line, K562 *(open triangle)*, and lysis of T2 cells pulsed with HBV *(open circle)* served as background controls. Lysis of the MCF-7 breast cancer cell line *(solid diamond)* was also evaluated. Data are representative from donors 5, 4, and 1, respectively. *Error bars* represent the mean ± SEM of triplicate samples ($*p < 0.05$)

capacity to elicit IFN-γ from CTL lines compared with APL 2 (specific responses detected in 5/5 and 1/5 individuals, respectively). These APL-specific CTLs also cross-react with wild-type peptide, as demonstrated by IFN-γ ELISpot assays. APL-specific CTL lines lyse target cells pulsed with wild-type E75. E75-specific CTLs were also activated by APL 5, suggesting that cells activated in vivo by tumor-derived wild-type peptide, cross-react with, and are stimulated by, Her-2/*neu* APLs. In a therapeutic setting, CTLs that have a low affinity for wild-type peptide or that are partially anergic could be activated by Her-2/*neu* APLs.

APL 2 (**FLFGSLALF**) contains two amino acid modifications different from wild-type E75. Although these substitutions resulted in increased affinity for cell surface HLA-A*0201 molecules compared with wild-type E75, APL 2 proved to be inefficient at generating CTL lines from four of five individuals. However, APL 2–specific CTLs from the single responsive donor retained recognition of wild-type peptide as determined by IFN-γ production and lysis of target cells pulsed with wild-type E75. Since positions one and two of a nonamer peptide are buried in the HLA peptide-binding cleft, these residues are thought to be solely involved in HLA contact and may not affect TCR binding. Additionally, the isoleucine (Iso^2) to leucine (Leu^2) substitution was a subtle modification, as these amino acids are very similar in composition. Our results with APL 2 illustrate that peptides may possess increased affinity for HLA class I molecules compared with the wild-type peptide, but affinity alone is insufficient in rational design of a highly immunogenic peptide capable of T-cell activation.

The rational design of APL 5 (**FLFESLAFL**) also resulted in an increased affinity for HLA-A*0201 molecules compared with wild-type E75. In contrast to APL 2, APL 5 elicited peptide-specific responses from all five individuals tested. CTLs raised on APL 5 reacted most strongly to challenge with the same peptide and cross-reactivity with E75 was also observed when APL 5–specific CTLs were challenged with wild-type peptide. It has been suggested that this weak MHC binding is due to a lack of interaction with the center of the peptide [28]. The increase in the affinity of APL 5 for the HLA-A2 molecule may result from interaction of the center of the peptide (residue 4) with the HLA, resulting in the presentation of a peptide with more favorable TCR-binding characteristics compared with wild-type peptide.

Molecular modeling of the HLA-A2 molecule complexed with E75 APL provided further insight into the differences of peptide presentation by the HLA-A2 molecule to TCR. The side chain of the Glu^4 residue in APL 5 appear to be extending out of the peptide-binding cleft, and may participate in the interaction with the TCR upon ligation of the TCR with the peptide-MHC complex. Therefore, increased peptide immunogenicity is likely due to the large side chain that interacts with the outer edge of the helix, allowing TCR contact. The wild-type E75 peptide and APL 2 contain a Gly^4 residue, which is smaller in size compared with glutamic acid, and may not interact with the TCR. APL 2 differs from E75 by only the first two amino acids of the peptide, yet E75 was better able to stimulate CTLs than APL 2. Loss of CTL reactivity indicated that while the modifications introduced into APL 2 yielded a peptide with an increased affinity for HLA-A2 antigen, the conformation of the peptide/HLA complex was not favorable for TCR recognition. Studies have also demonstrated that substitutions introduced into anchor positions can alter residues involved in TCR binding [29]. While it is difficult to provide a concrete explanation for this finding,

we might conclude that high affinity peptide/HLA complexes do not always translate to highly efficient cellular stimulators.

Although we observed minimal tumor cell lysis by our APL-specific CTL, one possible reason we could not demonstrate strong lysis of tumor cells was reported by Keogh et al. [30]. They found that CTL specific for APL, with more than one amino acid substitution compared with the wild-type peptide, were less frequently able to recognize the parental peptide. Several groups have also not been able to demonstrate lysis of tumor cells by peptide-specific CTLs [31, 32]. Additionally, low peptide density on the surface of the tumor cells may provide an explanation for the absence of tumor cell lysis. Zaks et al. [33] demonstrated that E75-specific CTLs isolated from patients immunized with E75 were unable to lyse tumor cells that expressed the Her-2/*neu* antigen. Similar to our findings, these CTLs lysed T2 cells pulsed with the E75 peptide. While the Her-2/*neu* antigen is overexpressed on tumor cells, the E75 peptide may not be present at high enough levels for TCR recognition. Studies have shown that increasing the peptide density on melanoma cells, via the addition of an exogenous melanoma-specific peptide (g209 2M), resulted in an enhanced sensitivity to peptide-specific CTLs [34]. We pulsed MCF-7 tumor cells that overexpressed the Her-2/*neu* antigen (MCF-7-2/18) with the E75 wild-type peptide and observed minimal lysis. It is possible that these cells secrete immunosuppressive factors that inhibit T-cell effector functions.

PBMCs stimulated for several rounds with wild-type E75 (KIFGSLAFL) demonstrated E75 specificity when challenged with T2 cells pulsed with E75 as determined by IFN-γ ELISpot assays. These E75-specific PBMCs were also activated by APL 5, suggesting that in vivo, existing anti-wild-type CTLs possess cross-reactive recognition of Her-2/*neu* APLs.

These findings indicate that the introduction of preferred amino acid residues into the Her-2/*neu* E75 peptide results in an increased affinity for the HLA-A*0201 allele compared with the wild-type peptide and may (1) activate existing CTLs specific for Her-2/*neu* and (2) elicit CTLs de novo that cross-react with wild-type peptide. Continuing studies are investigating CTL responses to APLs by wild-type anti-Her-2/*neu* CTLs from patients with Her-2/*neu*-positive tumors. These results will reveal if Her-2/*neu* APLs are capable of rescuing weakly responding or anergic CTLs in cancer patients.

Acknowledgements We are grateful to Dr Sue Roberts for her contributions to the molecular modeling of HLA-A2/peptide complexes. This study was supported by a National Cancer Institute grant, RO1 CA94852.

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Prevention (medical)

From Wikipedia, the free encyclopedia

In medicine, **prevention** is any activity which reduces the burden of mortality or morbidity from disease. This takes place at primary, secondary and tertiary prevention levels.

1. **Primary** prevention avoids the development of a disease. Most population-based health promotion activities are primary preventive measures.
2. **Secondary prevention** activities are aimed at early disease detection, thereby increasing opportunities for interventions to prevent progression of the disease and emergence of symptoms.
3. **Tertiary prevention** reduces the negative impact of an already established disease by restoring function and reducing disease-related complications.

Prevention of substance use

In the area of substance-related harms, a number of prevention typologies have been proposed.

Gordon (1987) in the area of disease prevention, and later Kumpfer and Baxley (1997) in the area of substance use proposed a three-tiered preventive intervention classification system: universal, selective and indicated prevention. Amongst others, this typology has gained favour and is used by a.o. the US Institute of Medicine, the NIDA and the European Monitoring Centre for Drugs and Drug Addiction.

1. **Universal prevention** addresses the entire population (national, local community, school, district) and aim to prevent or delay the abuse of alcohol, tobacco, and other drugs. All individuals, without screening, are provided with information and skills necessary to prevent the problem.
2. **Selective prevention** focuses on groups whose risk of developing problems of alcohol abuse or dependence is above average. The subgroups may be distinguished by characteristics such as age, gender, family history, or economic status. For example, drug campaigns in recreational settings.
3. **Indicated prevention** involves a screening process, and aims to identify individuals who exhibit early signs of substance abuse and other problem behaviours. Identifiers may include falling grades among students, known problem consumption or conduct disorders, alienation from parents, school, and positive peer groups etc.

Outside the scope of this three-tier model is **Environmental prevention**. Environmental prevention approaches are typically managed at the regulatory or community level, and focus on interventions to deter drug consumption. Prohibition and bans (e.g. smoking workplace bans, alcohol advertising bans) may be viewed as the ultimate environmental restriction. However, in practice environmental preventions programmes embrace various initiatives at the *macro* and *micro* level, from government monopolies for alcohol sales, through roadside sobriety or drug tests, worker/pupil/student drug testing, increased policing in sensitive settings (near schools, at rock festivals), and legislative guidelines aimed at precipitating punishments (warnings, penalties, fines).

References

- Gordon, R. (1987), 'An operational classification of disease prevention', in Steinberg, J. A. and Silverman, M. M. (eds.), Preventing Mental Disorders, Rockville, MD: U.S. Department of Health and Human Services, 1987.

Appendix C

- Kumpfer, K. L., and Baxley, G. B. (1997), 'Drug abuse prevention: What works?', National Institute on Drug Abuse, Rockville.

See also

- Alliance for Consumer Education
- Preventive medicine

Alliance for Consumer Education http://www.stopgerms.org/

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